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SCHAFER, ELISABETH A.

THE EFFECT OF DIETARY ALPHA-TOCOPHEROL ON PULMONARY FATTY ACIDS AND PROSTAGLANDINS IN RATS FED EXCESS LINOLEIC ACID

Iowa State University PH.D. 1980

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Effect of dietary α-tocopherol on pulmonary fatty acids and prostaglandins in rats fed excess linoleic acid

by

Elisabeth A. Schafer

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY
Major: Nutrition

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1980

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ARGG	Anti-rabbit gamma globulin
CE	Cholesteryl ester
DPPC	Dipalmitoyl-phosphatidylcholine
EFA	Essential fatty acid
EFAD	Essential fatty acid deficient or essential fatty acid deficiency
FA	Fatty acid
FER	Food efficiency ratio
HCO	Hydrogenated coconut oil
KPi	Potassium phosphate
NRS	Normal rabbit serum
PBS	Phosphate-buffered saline solution
PG	Prostaglandin
PL	Phospholipid
P/S	Polyunsaturated fatty acids/saturated fatty acids
PUFA	Polyunsaturated fatty acids
RBC	Red blood cell or erythrocyte
RIA	Radioimmunoassay
SAS	Statistical Analysis System
SO	Safflower oil
TG	Triglyceride
TPN	Total parenteral nutrition
T/T	Triene:tetraene or C20:3ω9/C20:4ω6
T/T + D	C20:3ω9/C20:4ω6 + C18:2ω6, triene/tetraene + diene

INTRODUCTION

Any abrupt change in diet may induce unexpected consequences. Never yet in nutrition have we been able to isolate one nutrient from all the others in its metabolic effects. Neither a significant increase nor decrease in intake of a single nutrient will have uncomplicated, totally predictable effects. An increased intake of one nutrient will influence the requirement for other selected nutrients. The host of interrelationships among nutrients complicates attempts to ameliorate a single physical need by a unique dietary alteration. Such is the case with fat-altered diets.

Since 1952 there have been repeated reports that dietary polyunsaturated fat (PUFA) is associated with lowered serum cholesterol (Kinsell et al. 1952, Ahrens et al. 1954, Hegsted et al. 1965, Grundy and Ahrens 1970). These reports held great promise for a dietary means of protection from cardiovascular heart disease, one of the major medical concerns of developed nations. Prompted by these results, the American Heart Association and the U.S. Senate Select Committee on Nutrition and Human Needs have recommended that we increase intake of PUFA. Recent dietary guidelines issued by the USDA and DHEW suggest reduction of saturated fat but not an increase in PUFA (U.S.D.A. and D.H.E.W. 1980). Many have been convinced to alter their diets radically. Consumption of PUFA in the U.S. has risen steadily (Rizek et al. 1974). This dietary change has occurred, however, without regard to the other effects such a diet may produce. The benefits of a high PUFA diet are not entirely clear, for there are conflicting results. Not all re-

searchers have been able to reduce serum cholesterol by raising dietary PUFA (Nichols et al. 1976). Jackson et al. (1978) have provided a comprehensive review of the contradictory evidence and some reasons for the diverse results.

Fear of cardiovascular heart disease is not the only impetus for changes towards diets relatively rich in PUFA. Patients maintained on long-term total parenteral nutrition (TPN) must receive essential fatty acids (EFA) in order to avoid manifestations of deficiency, and the lipid emulsions commonly used in such circumstances have P/S ratios of 7.3 and over (Silberman et al. 1977). Recent observations on cystic fibrosis patients suggest that at least part of the disease is due to an error in EFA metabolism (Campbell et al. 1976, Chase 1976). Rosenlund et al. (1977) and Elliott (1976) have fed increased levels of maize oil and soybean oil, respectively, with noticeable improvement in cystic fibrosis patients.

Unfortunately, there is mounting evidence of danger in high PUFA diets. Linoleic acid appears to accelerate tumor growth (Rao and Abraham 1976, Hopkins and West 1977b). Pinckney (1973) observed premature skin aging and a ruptured spleen from excessive ingestion of PUFA. Heng (1977) showed alteration in the structure of cardiac muscle and in its distensibility related to unsaturation of dietary fat, while others have observed increased incidence of respiratory distress in laboratory animals on high PUFA diets (Richards 1979). Rats aged 6 to 9 months fed mixed fats performed significantly better in a discrimination learning situation than rats fed 20% safflower oil. Moreover, the latter rats appeared to have a reduced lifespan (Harman et al. 1976).

Higgins (1979) reported decreased growth in young rats fed 38% of calories as safflower oil. Furthermore, it is believed that increasing the PUFA intake increases the dietary requirement for vitamin E (Horwitt 1962, Jager 1972, Harris and Embree 1963).

Dietary lipid is known to alter tissue lipid composition. High PUFA intake enriches tissues with PUFA (Danon et al. 1975, Carroll 1965). The consequences whether beneficial or dangerous are not yet fully known. Membrane structure and functional characteristics will certainly be affected, for polyunsaturated fatty acids (PUFAs) are vital components of mammalian membranes (Metzler 1977).

A major use of PUFA in the body is to serve as substrate for prostaglandin (PG) synthesis. The PGs have been observed in nearly all tissues and appear to be potent local regulators of varied functions: vasoconstriction, vasodilation, smooth muscle contraction and relaxation, bronchial dilation and constriction, platelet aggregation, lipolysis, reactions mediated by level of cyclic AMP, neural transmission, inflammation, intraocular pressure (Karim and Hillier 1972). There are a host of different PGs, all related in structure, being derived from the 20-carbon prostanoic acid skeleton but differing in double bond placement, side-chain substituents, and placement of hydroxyl and keto groups. These varying PGs frequently have opposing functions (Mathe et al. 1977). It is probably the balance among varying PG forms that allows fine, immediate control of localized events. Researchers have recently established that dietary intake of PUFA is associated with altered synthesis of PGs (Mathias and Dupont 1979). Whether the change in prostaglandin synthetic potential is the mechanism whereby dietary PUFA exerts its

influence is not fully established but currently is an accepted, working hypothesis.

The purpose of this work is to explore the interrelationships of high PUFA diets and dietary α-tocopherol, the most common form of vitamin E. We propose to test the hypothesis that (1) increased intakes of PUFA may produce a secondary vitamin E deficiency and, as a corollary, (2) that increasing intakes of α-tocopherol may ameliorate certain of the ill effects of high PUFA diets, specifically by altering the tissue level of PG precursor fatty acids and by altering tissue PG synthetic rate.

REVIEW OF LITERATURE

Fatty Acid Composition

Livestock producers were probably the first to observe that diet might influence the lipid composition of animal tissues. While pigs fed starchy cereals had hard adipose, those fed peanut-meal had soft adipose tissue (Holman 1964). When controlled nutritional studies regarding fat source became a reality with advancing technology, it was recognized that dietary fatty acid patterns could be reflected in tissue fatty acid patterns. Early studies focused on serum and adipose. Hirsch et al. (1960) reported that serum lipids in humans showed an increased linoleate content in response to a corn oil diet. They also observed an effect in adipose tissue but believed it to be a slow, gradual response. Repeated observations by many researchers confirmed the effect of dietary lipid on serum and adipose. Indeed, the fatty acid composition of serum and adipose was strongly representative of dietary lipid in laboratory animals (Leat 1963, Holman 1964, Carroll 1965) and in humans (Dayton et al. 1966).

Veerkamp et al. (1962) made extensive analyses of the fatty acids of lipid fractions of various tissues from several species. Dietary variables were not included in the experiment. The rats were given a commercial diet which was 6% fat (51% linoleic acid). The reported fatty acid patterns of neutral lipids in depot fat, liver, kidney, and lung can serve as useful references when considering the effect of varying dietary fat.

As research results have accumulated, we have found comparisons of corn oil, safflower oil, soybean oil, beef tallow, lard, cod liver oil, and butter fat in respect to their influence on serum and adipose fatty acid compositions. Metabolic studies in adolescent boys comparing safflower oil and beef tallow revealed statistically significant differences in C16:1, C18:1, and C18:2ω6 in serum lipids. When comparing corn oil and butter oil, researchers again found significant differences in serum oleate and linoleate. Corn oil and safflower oil, rich sources of linoleate, were both capable of elevating serum linoleate (Kies et al. 1978).

Another investigator compared safflower oil and coconut oil combined to produce varying P/S ratios and found that adipose tissue predicted the lipid composition of diet (Higgins 1979). A five-year study in elderly men compared the response of serum and adipose to a conventional diet and a diet in which unsaturated fat was substituted for saturated fat. Both diets contained fat at 40% of calories, which approximates current American and European intakes (Dayton et al. 1966). On the high polyunsaturated fat diet, linoleic acid increased significantly in all serum lipid fractions. The level of arachidonic acid rose slightly in triglycerides but was unchanged in other fractions, while palmitate and stearate decreased in cholesteryl esters, phospholipids, and triglycerides. The linoleic acid content of adipose rose from 11% of total fatty acids at the initiation of the experiment to 32% at the termination five years later.

So well established was the correlation of dietary and adipose fatty acid patterns that Beynan et al. (1980) proposed a direct

mathematical relationship between them. In fact, they asserted that measurement of the fatty acids of adipose may be used to identify whether or not individuals are faithfully adhering to recommended diets.

Research has also been directed at other tissues and organs to elucidate the role of dietary fat in altering their composition. Carroll noted in a review paper (1965) that many studies of serum and liver had been conducted. Today we may add that there is also information on brain, kidney, heart, skeletal muscle, adrenal gland, erythrocytes, platelets, and retina. Only a few investigators have explored fatty acid composition of lung. Liver, serum, and adipose, due to their pivotal position in lipid transport, clearance, synthesis, and storage reflected, as expected, the influence of dietary lipid. Brain, although the contribution of dietary fat thereto was limited, also showed significant changes (Carroll 1965). After feeding cod liver oil to rats, Rieckehoff et al. (1949) found that the lipid of the heart was similar in fatty acid composition to the oil fed. In rat muscle and liver dietary corn oil produced increased levels of linoleate, while a beef fat diet produced increased levels of oleate (Gurr et al. 1976). In a study of the effect of arachidonate and dihomo-γ-linolenate on the fatty acid composition of plasma and tissues, researchers concluded that the arachidonate content of tissues could be substantially increased by a high dietary intake of arachidonate. The same phenomenon occurred with dihomo-γ-linolenate (Danon et al. 1975). As the experiment was designed, the animals ate a commercial laboratory chow ad libitum and were given 300 mg ethyl arachidonate daily (or ethyl dihomo-γ-linolenate) by stomach tube. Arachidonate is generally present

in the diet in small amounts. Thus, the rats' ingestion of 300 mg daily, which is approximately 5% of calories, while appearing on one hand to be a high arachidonate intake, is on the other hand quite modest by comparison to linoleate intakes at 10-16% of calories on vegetable oil diets. Enrichment of all major lipid classes occurred — triglycerides, cholesteryl esters, phospholipids, and free fatty acids. Further studies of the metabolism of dihomo-γ-linolenate showed that oral administration of this fatty acid resulted in its incorporation in tissue lipids (Hassam and Crawford 1978b).

The fatty acid pattern of the triglyceride fraction represents dietary fat to the greatest extent, while the phospholipid fraction is least variable (Higgins 1979). Cholesteryl ester fatty acid response to diet varied with the tissue; hepatic CE was more sensitive to dietary fatty acid composition than was plasma CE. Pulmonary nonphospholipid fraction was more responsive to dietary fat alterations than were cardiac and renal nonphospholipid fractions. Pulmonary phospholipid P/S ratio varied from 0.41 to 0.45 when dietary P/S ratio was varied from 0.1 to 8.6. Thus, the phospholipid fraction of lung hardly altered despite major dietary fat alterations (Higgins 1979). Fine et al. (1980) confirmed the fact that platelet phospholipid fatty acid composition was unaffected by P/S ratios from 0.4 to 5.5. Those tissues containing much triglyceride will exhibit the greatest compositional response to dietary fat profile.

Research directed at refining our perceptions has produced evidence that dietary fatty acids have not only macro but also micro effects on tissue lipid composition. Using mice, Tsang et al. (1980) found

lymphocyte lipids reflected the FA composition of the diet after only two weeks. Not only organs and cells but also subcellular organelles may show lipid changes. Witting et al. (1961) showed the fatty acid composition of rat hepatic mitochondrial lipids was readily altered by dietary fatty acid variations. Depending on diet, fatty acids could readily displace one another and accumulate in brain mitochondria, erythrocytes, and heart tissue (Century et al. 1963). Hepatocyte plasma membranes contained elevated linoleate and arachidonate when the diet was high in PUFA (Hopkins and West 1977a).

Pulmonary fatty acid composition

Pulmonary fatty acids have had less attention than those of other organs and tissues. It is unfortunate that investigators of lipid metabolism have so often overlooked the lung while focusing on other organs. Although it is true that the total lipid content of lung tissue is low, nevertheless pulmonary lipid metabolism is highly active. The lung engages in vital lipogenesis for the surfactant; free fatty acids are rapidly esterified; fatty acid oxidation is swift in lung. The lung has been called "a titan of lipid metabolism" (Tierney 1974). In fact, due to a limited use of carbohydrate by adult lung, lipids may be the major source of acetyl-CoA in pulmonary mitochondria.

Much research on lung lipids has dealt with elucidation of the composition and chemistry of pulmonary surfactant. The pressure on alveoli is so great that the air sacs would collapse were it not for the presence of surface-active materials that reduce alveolar interfacial tension. Frosolono et al. (1970) reported successful isolation and

characterization of the surfactant fraction of lung. Identified as dipalmitoyl-phosphatidylcholine (DPPC), it was shown to consist of 68% palmitate, 7% palmitoleate, 7% stearate, 8% linoleate, and 8% an unidentified fatty acid.

Observations of the unusually high pulmonary content of saturated 16- and 18-carbon fatty acids compared to other mammalian constituents led to further purifications and analyses of the surface-active material. King (1974) reported the composition to be 50% fully saturated phosphatidyl choline (primarily palmitate), 25% monoenoic phosphatidylcholine, and small amounts of other phospholipids, proteins, phosphatides, and cholesterol. The saturated phosphatidylcholine lowers alveolar interfacial tension, while the monoenoic phosphatidylcholine and cholesterol provide increased molecular mobility and adsorption to the alveolar interface (King 1974). Repeated analyses of fatty acid composition of pulmonary surfactant showed very high palmitic acid content (Sanders and Longmore 1975). Therefore, when total lung lipids were analyzed, a striking feature was the contribution of surfactant and the consequent high proportion of saturated fatty acids (Kehrer and Autor 1978, Donovan et al. 1977).

Essential Fatty Acids

Of particular interest in the study of dietary fat alterations and their effects on the body has been the role of essential fatty acids (EFAs) and the occurrence of essential fatty acid deficiency. Burr and Burr (1929) first recognized the essentiality of certain fats in the

diet of laboratory rats. The deficiency symptoms they observed were scaly skin lesions, necrosis of the tail, impaired growth, and renal degeneration. Further studies identified linoleic acid (C18:2ω6) as the critical dietary component. Over the years there has been indecision among nutritionists as to the precise fatty acids to be considered dietary essentials. Of linoleic acid, there is no doubt. In 1974 the National Research Council identified arachidonic acid as essential. In 1980 the National Research Council identified linoleate as the primary dietary EFA and arachidonate as a minor contributor. Very little arachidonate occurs in the average diet (Brignoli et al. 1976, Weihrauch et al. 1977); and humans, rats, and other species are capable of synthesizing arachidonate from linoleate. Until recently, it was believed that the domestic cat lacked the Δ-6 desaturase enzyme necessary for arachidonate synthesis (Rivers and Hassam 1975). However, Stephan and Hayes (1978) presented evidence that even the cat can thrive on a diet whose only fat source is linoleate.

The desaturase enzyme system appears to be very active under "normal" conditions, although many factors influencing its activity have been identified – (1) insulin, (2) dietary protein, (3) dietary carbohydrate, (4) quantity of endoplasmic reticulum, (5) relative amounts of competitive fatty acid substrates (Brenner 1974), and (6) dietary iron (Rao et al. 1980). No doubt, many more factors also influence the desaturase system. Dietary arachidonate has been shown to express an essential fatty acid potency that is two to three times that of linoleate, as measured by its ability to reverse EFA deficiency symptoms (Crawford et al. 1978).

Much discussion has occurred concerning the dietary necessity of linolenate or the ω3 fatty acid family. Takehisa and Kimura (1977) reported dermal symptoms in rats were not prevented by dietary linolenic acid. The rats showed epidermal thickening and abnormally increased phospholipid activity in the skin, classic signs of EFA deficiency. Leat and Northrop (1979) also failed to show adequate protection by linolenate. Although dietary linolenate was capable of supporting growth and gestation, its addition to the diet resulted in severely impaired parturition. The explanation was that linolenate was incapable of serving as substrate for PG synthesis, and PGs were necessary for successful parturition. These studies, however, do not prove that linolenate is nonessential, only that it is inadequate in isolation from other dietary fatty acids. Crawford et al. are major exponents of the belief that α-linolenic acid or other ω3-fatty acids cannot be dismissed as nonessential (1978). They supported their contention with comparisons of the triene:tetraene (T/T) ratios in blood phospholipids of infants. Infants fed cow's milk (very low in linolenate) had T/T = 0.2-0.3. On the other hand, infants fed human milk (relatively high in linolenate) had T/T = 0.05. Crawford and associates believed these results, coupled with the presence of ω3-fatty acids in brain and retinyl lipids were compelling reasons not to neglect linolenate. Tinoco et al. (1978), having observed α-linolenic acid in rat and human retinal lipids, rat synaptosomal membranes, and human cerebral gray matter, agreed; however, attempts to provoke α-linolenate deficiency apparently failed. The authors reported that withholding dietary α-linolenate had no effect on growth, reproduction,

behavior, or general fatty acid composition in any lipid class in any tissue.

A further problem in assessment of EFA requirement has been quantitative. If we assume linoleate as the major EFA, how much is needed? The generally accepted level, based on ability to prevent plasma and hepatic T/T ratio from rising above 0.4, has been 1-2% of calories as linoleate (Alfin-Slater and Aftergood 1968, Holman 1970). In a study with young female rats, Hassam and Crawford (1978a) observed their food efficiency did not improve until linoleic acid intake reached 4% of dietary energy. From a study of human adult males on total parenteral nutrition (TPN), Richardson and Sgoutas (1975) also concluded the requirement should be set at 4% of calories from linoleate. The 1980 edition of Recommended Dietary Allowances suggests 8-10% of calories as linoleate may be beneficial to at least a portion of the United States population (National Research Council 1980).

For the purposes of our study, we considered EFA deficiency to be a complete dietary absence of α-linolenate, linoleate, and arachidonate.

Experimental EFAD diets

For laboratory observations two basic dietary regimens have been employed to induce EFA deficiency: (1) the fat-free diet, and (2) the saturated fat diet. In the fat-free method researchers rigorously excluded any fat from the diet, providing usually 20% of calories as protein and the remaining energy from sucrose. Due to generous body stores of EFA, this diet required lengthy feeding to produce either clinical or biochemical symptoms of EFA deficiency – in mice 8 weeks

(DeWille et al. 1979), in rats 15 weeks (Bohles et al. 1976) to 28 weeks (Kaa 1976). Other investigators felt the only way to deplete body EFA pools was to feed a fat-free diet to the mother and so prevent the offspring from accumulating EFA from birth (Jonsson et al. 1979).

The second experimental deficiency diet contained varying amounts of fat (2% to 30% of dietary energy as fat) but rigorously excluded polyunsaturated fatty acids. Thus, the dietary fat was necessarily rich in saturated fatty acids or medium and short chain fatty acids. Hydrogenated coconut oil (Williams et al. 1972), medium chain triglycerides (Hirono et al. 1977), and hydrogenated fish oil (Kaa 1976) all were used. Comparisons of the experimental diets repeatedly showed that the presence of saturated fat in the diet accelerated and exacerbated the deficient state (Deuel et al. 1955, Alfin-Slater et al. 1965, Williams et al. 1972). Williams et al. (1972) compared 25% hydrogenated coconut oil with a fat-free diet. The saturated fat diet resulted in more severe growth depression and dermal lesions than were observed on the fat-free diet, although the fatty acid patterns in tissues were not markedly different. Both diets produced the characteristic EFA deficient fatty acid profiles. The authors concluded that the hydrogenated coconut oil either interfered with EFA utilization or had an unknown metabolic effect. Although both fat-free and saturated fat diets produced EFA deficiency, they were not identical in effect. There was evidence that hepatic enzymes adapted to a fat-free diet by increasing lipogenic activity (Romsos and Leveille 1974). The presence of fat in the diet, any fat, reduced this enzymatic activity to control levels (Tepperman and Tepperman 1970). Similarly, presence of fat in the diet may have

prevented a decrease in activity of lipolytic enzymes, and thus, the more rapid appearance of EFA deficiency resulted from continued oxidation of EFAs rather than conservation. The fat-free diet may be criticized on two accounts. A fat-free diet brings about sharp alterations in lipid-metabolizing enzymatic activity. A fat-free diet is of lower caloric density than a diet containing a moderate level of fat. Low caloric density might make it difficult for an animal to eat adequate quantities of food to maintain body weight.

Investigations dealing with hydrogenated coconut oil (HCO) diets have also considered the effects of varying levels of casein. Rats were fed either 25% HCO and 20% casein or 25% HCO and 25% casein. After four weeks the group on the higher casein diet had maintained body weight better than the other group, although after eight weeks, weight difference between the two groups had disappeared. Thus, the higher casein diet appeared to provide moderate protection against the growth depression usually seen on EFA deficient diets (Williams et al. 1972). Hill and Holman (1980) varied the casein in EFA deficient diets from 5 to 40%. Dermal signs increased in severity at levels of protein over 30%, while the T/T ratio of liver phospholipid was most elevated at low protein levels. Body weight increased sharply when protein content of the diet was raised from 5 to 20%, but there was little improvement in body weight at higher protein intakes. Thus, dietary protein is an important variable in expression of EFA deficiency.

Fatty acid interactions

The nutritional and metabolic interrelationships among fatty acids prevent simple considerations of isolated fatty acids. Holman (1964), Mohrhauer et al. (1967) and Mead (1961) were among the leaders in elucidating polyenoic fatty acid interactions. Eighteen-carbon fatty acids occur naturally in three families – ω3, ω6, and ω9 – according to position of the C-C double bond nearest to the methyl terminus. All three families undergo desaturation and elongation, competing for the same enzymatic system. Dependent upon the initial fatty acid, the end products will differ. Figure 1 shows the three fatty acid families and their metabolism scheme. As will be noted, the Δ6 desaturase can act upon oleate, linoleate, and α-linolenate; and this desaturation may occur prior to elongation. Since all three substrates compete, the production of C20:3ω9 can be suppressed by the presence of C18:2ω6. Thus, in EFA deficiency, when C18:2ω6 is lacking, appearance of C20:3ω9 is to be expected. Holman (1964) clearly showed that depression of the metabolites of one family is occasioned by feeding members of another family. Brenner (1974) reported that C20:4ω6 inhibited the conversion of C18:1ω9 to C20:3ω9. Saturated fatty acids had no inhibitory effect. Alpha-linolenate was a stronger competitor than oleate, having a smaller Km. Therefore, presence of any ω3 or ω6 fatty acid suppressed synthesis of C20:3ω9. The rate-limiting enzyme of the entire desaturation-elongation system was believed to be the Δ6-desaturase (Hassam and Crawford 1978b). Perhaps cystic fibrotics, who appear EFA deficient, although no C20:3ω9 is apparent, lack Δ6-desaturase (Rivers and Hassam

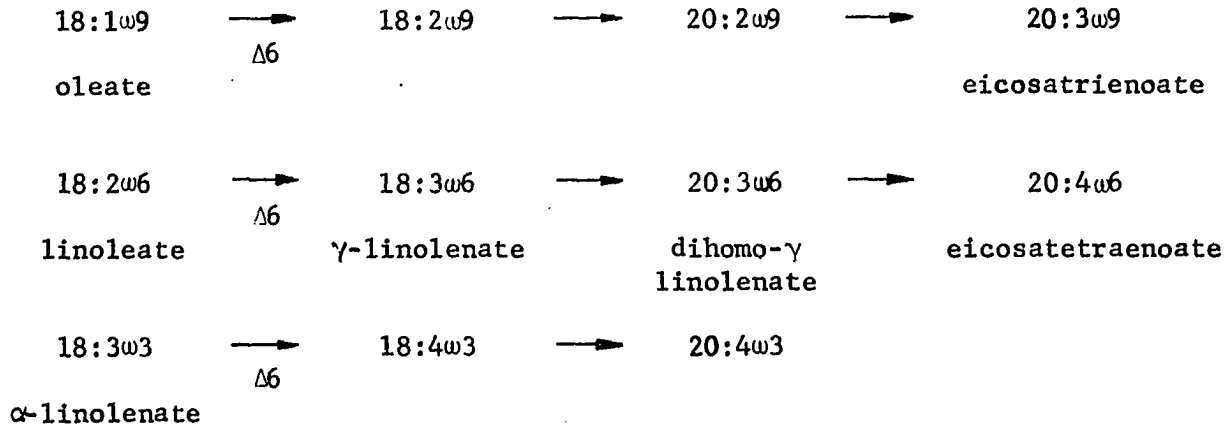

Figure 1. Desaturation and elongation of C18 fatty acids (after Mead 1961)

1975). A recent observation suggested that a fat-free, iron-sufficient diet stimulated Δ6-desaturase activity (Rao et al. 1980).

Importance of EFA deficiency

Although EFA deficiency was first reported over fifty years ago, there was but minor interest among researchers who believed it to be of little practical concern in humans. The deficiency was not recognized in general populations, humans had generous reserves of EFA, and the food supply provided more than adequate EFA. In the last decade, however, interest has burgeoned. Essential fatty acid deficiency is no longer an impossibility. The appearance of long-term TPN for surgical and other traumatized patients, of intravenous feeding of premature or other at-risk infants, and recognition of fat malabsorption syndromes have all mandated a fuller understanding of EFA deficiency. Richardson and Sgoutas (1975) reported EFA deficiency appeared in adults within only three weeks on TPN, and other researchers confirmed the reality of EFA deficiency today (Fleming et al. 1976, Wene et al. 1975). Biochemical

evidence of EFA deficiency could be detected in infants within two weeks, although dermal symptoms were slower to appear (O'Neill et al. 1977).

Consequences of EFA deficiency

The standard identification of EFA deficiency has been by the triene:tetraene (T/T) ratio proposed by Holman (1960). In normal serum and tissues one detects only traces of eicosatrienoic acid (C20:3ω9). Thus, the ratio of C20:3ω9 to C20:4ω6 is very small. In EFA deficiency, on the other hand, eicosatrienoic acid increases with progress of the deficiency, while the level of eicosatetraenoic acid (C20:4ω6) is believed to decrease. Thus, the T/T ratio rises. A T/T ratio greater than 0.4 is considered to signal EFA deficiency. The T/T ratio has been variously determined in plasma, liver, heart, and other tissues and from total lipids, phospholipids (PL), and other lipid classes. Depending on the method of determination and depth of deficiency, ratios were reported from 0.2 in blood PL (Crawford et al. 1978) to 1.6 in skin (Skolnik et al. 1977) to 2.9 in salivary gland (Alam and Alam 1978) to 3.4 in liver (Hassam and Crawford 1978a). Bohles et al. (1976) believed the T/T ratio of the cholesteryl ester (CE) or PL fraction of plasma was the most sensitive indicator of EFA intake. Triglyceride had a slow turnover rate. Total lipid, containing a large amount of triglyceride was, therefore, less responsive to dietary alterations.

With a resurgence of interest in EFA deficiency came reports of subtle consequences of the dietary lack. To the earlier and more

obvious clinical symptoms, researchers added a host of observations attributable to the EFA deficiency disease. DeWille et al. (1979) depleted mice of EFA for 70 days. At the end of 30 days, there was no difference in body weight, thymus, or spleen weights in EFA deficient or control animals. However, by 70 days the deficient mice had significantly reduced body weights, although their energy intake did not differ from control animals. Fatty livers have frequently been reported in EFA deficiency. Decreased hepatic secretion of triglycerides was suspected. Huang and Williams (1979) tested the postulate and found just the opposite. Their animals not only had fatty livers but also a twofold increase in hepatic triglyceride release. The authors speculated, therefore, that the hepatic lipidosis must be due to enhanced hepatic lipogenesis in the deficient state. Mitochondria were distorted and enlarged by lipid deposits in liver (Richardson and Sgoutas 1975) and in lung (Edmonds et al. 1975). Stephan and Hayes (1978) reported depressed appetite, mild anemia, and lowered plasma α-tocopherol in EFA deficient cats.

Mature male rats fed 10% of dietary energy as HCO were protected against oxygen toxicity as compared to rats fed either standard laboratory chow or a 10% cod liver oil diet (Kehrer and Autor 1978). Thus, not all the consequences of EFA deficiency are dire.

Dermal symptoms are late-appearing. O'Neill et al. (1977) recognized EFA deficiency in human infants biochemically long before observing hair loss, hair depigmentation, and poor wound healing. Jonsson et al. (1979) verified and quantified the slow rate of wound healing. Skolnik et al. (1977) reversed the deficiency effects on skin by topical application of safflower oil. Within 21 days not only had the T/T of

skin returned to normal, but also the T/T of serum had dropped prominently. Cutaneous treatment with safflower oil improved erythrocyte and plasma fatty acid patterns as well as skin symptoms, suggesting that in EFA deficiency the skin was highly permeable and oil readily absorbed (Bohles et al. 1976). Takehisa and Kimura (1977) reported an increased phospholipid synthetic activity in EFA deficient skin.

As has previously been implied, the fatty acid pattern of tissues was greatly altered when EFA was lacking in the diet. The typical change in FA pattern caused by EFA deficiency, no matter the tissue or the lipid fraction, involved a rise in C20:3ω9, C16:1, and C18:1 and a concomitant decline in C18:2ω6 and C20:4ω6 (Bohles et al. 1976, Holman 1964, Kehrer and Autor 1978). Drastic FA composition changes were reported in salivary gland (Alam and Alam 1978), endocrine system (Panos and Finerty 1954), oral palatal epithelium (Lekholm 1976), serum and adipose (Privett et al. 1965), liver (Peluffo et al. 1976, Fallani et al. 1976), lung (Kyriakides et al. 1976), brain (Sun 1972, Odutuga 1977), and erythrocytes (Hirono et al. 1977). Other lipids were also affected by the deficiency. Brain cell and membrane structures were shown to be disturbed while cerebroside and sphingomyelin content of brain was diminished (Odutuga 1977).

As might be expected, EFA deficiency has profound effects on enzymatic activities. Hazinski et al. (1975) found elevated pentose cycle activity in isolated adipocytes which was not reversible by the addition of the EFA metabolite prostaglandin E_1. Brivio-Haugland et al. (1976) speculated that membrane structure was altered so as to reduce binding affinity, change the number of receptor sites, or alter concentra-

tion of cofactors for enzyme activation.

Essential fatty acid deficient animals had a reduced inflammatory response (Denko 1976) and a reduced humoral immunity response (DeWille et al. 1979). Essential fatty acid deficient human infants had impaired platelet aggregation in response to ADP compared to other low birth-weight infants. Clinical hemorrhage occurred in 80% of EFA deficient infants (Friedman et al. 1977). Essential fatty acid deficiency was implicated in the occurrence of hypertension. Genetically hypertensive rats had a lower level of C20:4ω6 in platelets than normal rats, which suggests an increased requirement for EFA in hypertensives (Lehmann et al. 1977b).

The effects of EFA deficiency on lung are manifold. Not only did EFA depletion enhance survival from O_2 toxicity as previously reported, but also rat lung lipids reflected the typical EFA deficiency pattern (Kehrer and Autor 1978). Table 1 has a comparison of pulmonary fatty acid composition in EFA deficiency and sufficiency. Weanling male rats fed a fat-free diet for 24 weeks had reduced secretion of pulmonary surfactant and enlarged mitochondria with cristae packing the intramitochondrial space (Edmonds et al. 1975). Friedman and Rosenberg (1979) isolated human pulmonary surfactant for FA analysis and reported decreased palmitate and palmitoleate and increased oleate under conditions of EFA deficiency. It was a striking observation that although plasma palmitate rose during EFA deficiency, pulmonary surfactant palmitate dropped. This alteration in the FA composition of surfactant may impair its surface active qualities. Indeed, Hopkins et al. (1963)

Table 1. Fatty acid composition of mature rat lung lipids after 33 days on experimental diets (from Kehrer and Autor 1978).

Fatty acid	Triglyceride % of total FA		Phospholipid % of total FA	
	HCO[a]	CLO[b]	HCO	CLO
16:0	24.7	21.9	30.0	33.9
16:1	8.0	7.7	8.6	8.1
18:0	5.2	4.9	13.5	11.7
18:1	36.9	36.8	18.3	15.3
18:2	1.6	3.4	1.1	0.8
18:3	0	9.5	0	2.3
20:4	0.3	2.7	17.3	7.1

[a]HCO = hydrogenated coconut oil, 10% of calories.

[b]CLO = cod liver oil, 10% of calories.

reported that EFA deficiency caused a respiratory disease syndrome in chickens. Kyriakides et al. (1976) independently showed the palmitate content of pulmonary tissue to be decreased in EFA deficiency and the surfactant activity of lung lavage phosphatidylcholines to be impaired. Refeeding of 4% safflower oil reversed the effects. Gross lung morphology was not altered in EFA deficiency, although Type II alveolar cells were affected (Friedman and Rosenberg 1979).

As EFAs are precursors for prostaglandin (PG) formation, one would expect EFA deficiency to be apparent in PG metabolism. Indeed, explanations for the previously mentioned hypertension, impaired platelet aggregation, inflammatory and immune responses may rest in the PG

synthetic capacity of the appropriate organs and tissues. Friedman et al. (1978) reported a decrease in a PGE metabolite in urine in human infants with EFA deficiency. Prostaglandin content of brain was diminished and platelet PG formation reduced in EFA deficiency (Hassam et al. 1979). Alterations of kidney PG metabolism occurred (Van Dorp 1971). Ziboh et al. (1974) reported a decline in PG synthetic capability in EFA deficient animals. Attempting to identify the cause, they found that an unsaturated FA occurring in the lipids of EFA deficient rats directly inhibited the cyclooxygenase activity of sheep vesicular glands. This inhibiting fatty acid was probably C20:3ω9, and it appeared to act by competitive inhibition. The effect, however, was insufficient to explain fully the decrease of synthetic capacity in EFA deficient animal tissues.

Bailey (1977) showed that several types of cells were able to grow and metabolize normally in the absence of EFA, e.g., human skin epithelium, human fetal cells, and rat liver parenchyma cells. He concluded that EFAs were not required for membrane structural purposes and that perhaps their sole essential biological function was to serve as precursors for PGs. However, cell growth and reproduction in culture does not signify ability to function properly in the whole organism. Ziboh and Hsia (1972) found that treating EFA deficient rats with topical applications of PGE_2 cleared the dermal symptoms. Parnham et al. (1979) reported that not all the consequences of EFA deficiency could be relieved by PG supplementation. Hazinski et al. (1975) concluded that the metabolic effects of EFA deficiency were more complex than simply reduced levels of PGs, since in vitro addition of PGE_1 had no effect on

EFA deficient cells. The role of EFAs as precursors of the PGs is vital but is probably not the sole biological function of the EFAs.

Prostaglandins

Less than ten years after recognition of the EFAs came the discovery of a vaso-depressor, smooth-muscle-stimulating substance in human semen that von Euler (1935) called "prostaglandin." It was more than 25 years later before the structure of this new substance was determined, and that proved to be one of the most fruitful and provocative discoveries of nutritional biochemical science. For the prostaglandins were shown to be active metabolites of the EFAs. Prostaglandins have a wide distribution in mammalian tissues and in other animal species (Normura and Ogata 1976). Prostaglandins and their precursors and products participate in a host of physiological processes, usually mediating metabolic actions within the cells in which they are synthesized.

Prostaglandin synthesis

The details of PG biosynthesis are not yet fully described, although much progress has been made. The basic requirements for PG synthesis include: (1) the prostaglandin synthetase enzyme system – EC1.14.99.1; (2) twenty-carbon ω3 or ω6 fatty acids with multiple sites of unsaturation, (3) molecular O_2, and (4) cofactors (reducing agents). Prostaglandins of the one series are metabolic products of dihomo-γ-linolenic acid. Prostaglandins of the two series are metabolic products of

arachidonic acid. Figure 2 shows the relationships of the ω6 EFAs and their PG products.

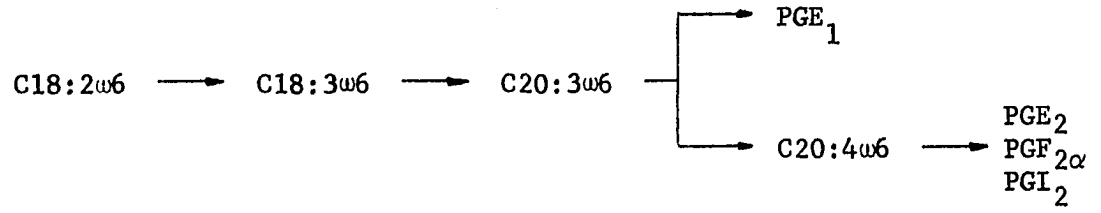

Figure 2. Generalized scheme of ω6 fatty acid conversion to prostaglandins

Fatty acid substrates for PG synthesis must be nonesterified. Because the PL fraction is rich in EFA, much attention has focused on phospholipase, and this enzyme has been considered rate-limiting (Russell et al. 1975), at least for the two series of PGs. Vane (1971) showed that nonsteroidal anti-inflammatory agents, e.g., aspirin and indomethacin, inhibit PG synthesis. Lands et al. (1973) showed that many antioxidants also inhibit PG synthesis.

PGs are rapidly metabolized, having a biological half-life in the circulation of a few minutes at most (Vane 1969). According to one estimate, more than 80% of the PGE_1 was metabolized on a single pass through the lung (Ferreira and Vane 1967). Golub et al. (1975) determined that 68% of the PGE_1 was removed from the circulation in one transit through the lung, while only 8% of the PGA_1 was removed.

Prostacyclin

Moncada and Vane (1977) reported the discovery of an endoperoxide intermediate in PG metabolism which was 20 to 30 times as potent as PGE_1 in inhibition of platelet aggregation. This substance, termed

prostacyclin or PGI_2, was formed exclusively in the vessel wall and was, in fact, the major route of endoperoxide metabolism in vessel walls. Observations showed the vessel microsomes did not use arachidonate to generate PGI_2. A new homeostatic mechanism was proposed. "Platelets attempting to stick to the vessel wall release endoperoxides which are then used by the endothelial cells to make PGI_2 which then repels the platelets and stops them from sticking" (Moncada and Vane 1977, p. 163). Under normal circumstances, the intact vessel wall protects itself from platelet deposition. However, vascular damage may impair production of the protective prostacyclin and lead to thrombosis.

Gryglewski et al. (1978) measured the effects of prostacyclin but not prostacyclin itself by bathing rabbit collagen strips with arterial and venous blood. They found PGI_2 was consistently higher in arterial than venous blood and concluded that the lung served an important endocrine function by continuously generating and releasing PGI_2 in vivo and thereby protecting against intraarterial thrombosis. Dembinska-Kiec et al. (1979) concluded from their observations that prostacyclin was the major arachidonate metabolite formed by normal lungs.

Relationship of PGs and diet

Prostaglandins have potent and complex effects in physiological systems. In many instances different PGs have opposing effects. The balance among PG forms is probably a mechanism for fine, immediate control of local events. Awareness of the multitude of physiological effects of the PGs led to concern over the levels of PGs in various

tissues. Control of PG biosynthesis appeared to be a method for regulating body functions. Availability of the precursor fatty acids by dietary alterations was of practical importance.

Prostaglandins and EFA deficiency

Under conditions of EFA deficiency, when precursor levels are low, one expects PG biosynthesis to be diminished. Vincent et al. (1974) reported that the inhibition of collagen-induced platelet aggregation by PGE_1 was considerably reduced in EFA deficient rats. However, there was no measurement of PGs. Others, reporting on EFA deficiency and PGs, implied reduced PG synthesis without actually measuring the PGs (Kupiecki and Weeks 1966, Hazinski et al. 1975, Ziboh and Hsia 1972). Kaa (1976) found that PGE_2 synthesis in renal medulla of EFA deficient rats actually increased. Parnham et al. (1979) measured endogenous PG production in EFA deficient rats using a bioassay of PGE on isolated rat stomach strip. As this method did not adequately differentiate PGs, it is difficult to evaluate the results. Nevertheless, the authors showed reduced endogenous PG in serum. Other reports also showed that an EFA deficient diet reduced PG synthesis (Van Dorp 1971, Weston and Johnston 1978, Christ and Nugteren 1970). After examining many batches of animals over time, Parnham et al. (1979) concluded that endogenous PG production was an important indicator of EFA status. Depressed PG synthesis in EFA deficiency could be due not only to diminished precursor availability but also to the competitive, inhibitory presence of C20:3ω9.

Prostaglandins and PUFA-rich diets

Conversely, were increasing levels of dietary EFA accompanied by increasing PG synthesis? In an early experiment on this problem, Silver et al. (1974) administered large doses of arachidonic acid to rabbits. The treatment resulted in sudden death of the animals due to thrombosis. Since it had previously been shown that an arachidonic acid metabolite, $PGF_{2\alpha}$, was an aggregatory agent, these results strongly suggested that altering the precursor fatty acid level could affect PG synthesis. Danon et al. (1975) reported that rat tissues could be enriched with PG fatty acid precursors. Ethyl arachidonate and ethyl-dihomo-γ-linolenate, fed by stomach tube, were incorporated into all lipid classes of the tissues observed, but especially into triglycerides. In the same year, Hwang et al. (1975) measured the PGs themselves after different dietary treatments. In a paper that is now a classic in the area, they reported that serum concentrations of PGE_1 and $PGF_{2\alpha}$ measured by radioimmunoassay (RIA) were higher in rats fed 20% corn oil than in those fed 20% beef tallow. Although the serum samples were not handled in a properly controlled manner at autopsy resulting in some variability, the results have been confirmed in many later experiments. By feeding trans-linoleate to rats for 12 weeks, Hwang and Kinsella (1978, 1979) brought about a decrease both in precursor fatty acids and in PG synthesis in arterial blood. Feeding linolenate, Hwang and Carroll (1980) confirmed that PG synthesis could be regulated by precursor fatty acid supply. Prostaglandin and arachidonic acid levels in serum were well correlated (Hwang et al. 1979). Meydani et al. (1978) found that lung PG synthetic potential was increased tenfold in rats on a high compared to a low-PUFA

diet. Mathias and Dupont (1979) varied the P/S ratio of a 20% fat diet between 0.4 and 5.5. No change in $PGF_{2\alpha}$ and PGE_2 in lung nor in ten-minute incubated serum occurred. However, if the P/S ratio of the diet were raised to 9, then serum PG levels increased dramatically. Dupont et al. (1978) varied dietary linoleate in graded levels from 0 through 30% of energy and measured venous serum PGE_1, PGE_2, and $PGF_{2\alpha}$. Increasing the dietary linoleate content from 5% to 30% produced a linear rise in PG synthesis rate for PGE_1 ($r = 0.86$) and $PGF_{2\alpha}$ ($r = 0.97$), but there was no observed alteration in PGE_2 synthesis. DeDeckere et al. (1979) fed rabbits and rats for four weeks sunflower seed oil, hydrogenated coconut oil, or lard. Prostaglandins were measured by gas chromatography and bioassay. The authors failed to see an effect of dietary fat on PG synthesis. Similarly, Fine et al. (1977) found that serum PGE_1 was not affected by the P/S ratio of the diet.

Thus, results are mixed. Not all researchers find a correlation between dietary EFA and PG synthesis or endogenous PG. Some of the discrepancy is due, no doubt, to differing methods of sample handling and differing analytical techniques.

Prostaglandins are present in tissue in minute amounts. Injury, manipulation, sample collection itself stimulate generation of prostanoids. During sample storage, PG synthesis continues unless inhibitor is added. Even small discrepancies in handling cause major differences in PGs measured. Mass spectroscopy is the preferred analytical method but has limited sample capacity. Radioimmunoassay can handle large numbers of samples but is subject to cross-reactivity and specificity problems. The use of standardized, controlled tissue handling and

similarity in analytical procedures is, therefore, essential for meaningful comparisons.

Vitamin E

Vitamin E and polyunsaturated fatty acids are intimately linked in the diet and in the body. The consensus holds that vitamin E requirement is not a simple, fixed value. While the dietary requirement may be influenced by age (Farrell et al. 1978), environmental influences, and toxic materials, the major determinant of vitamin E need is amount of PUFA in the diet (Harris and Embree 1963). Generally, the interrelationship of vitamin E and PUFA is expressed as a ratio of vitamin E (mg): PUFA (g). Vitamin E is a generic term applying to total tocopherols and including α, β, γ, δ forms. Alpha and gamma tocopherols are by far the most common in our food supply. Gamma tocopherol is present in vegetable oils in higher concentration than α-tocopherol (Bieri and Evarts 1975b), but γ-tocopherol has only 20% the biological activity of α-tocopherol (Bieri and Farrell 1976). For the purposes of the present discussion, vitamin E is used to indicate vitamin E activity and presumes inclusion of total tocopherols unless otherwise specified.

Vitamin E requirement

In the "Elgin" studies adult males who were maintained on a dietary E:PUFA ratio of 0.4 slowly developed vitamin E deficiency as manifested by erythrocyte hemolysis (Horwitt 1962). At an E:PUFA ratio equalling 0.9 patients remained free of undue hemolysis but incurred no reserve, for within one week of cessation of supplementation, they exhibited

abnormal hemolysis. Thus, the conclusion was that an E:PUFA ratio approximating 1.6 was more truly adequate. The E:PUFA ratios required for adequacy of vitamin E nutriture vary by species. Rats on a 15% corn oil diet appeared normal when the E:PUFA ratio was as low as 0.1 (Harris and Embree 1963). The "average" U.S. diet in 1960 had an E:PUFA ratio of 0.616. Because Harris and Embree (1963) were unaware of overt deficiency, they concluded 0.6 could serve as a reference ratio for adequacy of vitamin E supply. Any diet with an E:PUFA ratio less than 0.6 should be considered potentially deficient.

The use of ratios has been severely criticized as being rigid, simplistic, and unrealistic (Witting and Lee 1975a). In diets of 5% or 10% fat and graded levels of PUFA with no diet providing more than 2.75% of calories as PUFA, the levels of PUFA and α-tocopherol in rat plasma increased in parallel. This result suggested that increasing intake of PUFA was automatically accompanied by increased intake of vitamin E. However, when linoleate intake reached 5.5% of calories, plasma tocopherol began to drop (Bieri and Poukka 1970). Clearly a single ratio was not adequate over a range of PUFA intakes. Jager (1972) explored this same problem in detail and confirmed the conclusion. Using erythrocyte hemolysis in rats as the deficiency symptom, he calculated a shift in vitamin E requirement with changing linoleate intake. However, the relationship between the intakes of the two nutrients was not linear. As linoleate varied from 0 to 50 g/kg diet, the vitamin E requirement was fairly stable. As linoleate increased to higher intakes, the vitamin E requirement began to rise exponentially. Dietary linoleate at up to 7% of calories caused no increase in

vitamin E requirement, but when dietary linoleate was over 20% of calories, the vitamin E requirement was increased at least 50% (Jager 1972).

Dam (1962) showed that increased dietary PUFA increased vitamin E requirements. Moreover, excessive intakes of PUFA appeared to precipitate vitamin E deficiency symptoms. Erythrocytes from vitamin E deficient rabbits were not susceptible to dialuric acid peroxidative hemolysis until the rabbit diet was supplemented with arachidonate. Feeding PUFA to vitamin E depleted rabbits acutely initiated the onset of hemolysis and muscular dystrophy (Brin et al. 1974). Bunnell et al. (1975) fed a low vitamin E diet to men engaged in hard physical labor. One group, which received safflower oil, showed a sharp decline in plasma tocopherol levels from their normal 1.42 mg/100 ml to 0.5 mg/100 ml, the minimally adequate level. The men had no symptoms of physical or muscular weakness. In an animal study a threefold increase in dietary PUFA depleted tissue stores of vitamin E, reducing E:PUFA ratios 26-63% (Bieri et al. 1978).

Vitamin E requirement may be influenced by tissue PUFA levels (Horwitt 1962). At low levels of intake (1-2% of calories), linoleate and other ω6 fatty acids concentrated largely in the PL. A maximum level was reached beyond which the composition of PL no longer altered significantly. Thus, at higher dietary levels of linoleate, accumulation occurred in the other lipid compartments. Both phospholipids and neutral lipids, therefore, were important in determining vitamin E requirement (Witting 1972). The vitamin E requirement was related to tissue PUFA. But the tissue PUFA was related to the dietary PUFA.

The general trend to increased consumption of PUFA was reflected in higher levels of PUFA in the adipose of female college students as compared to the early 1960's (Witting and Lee 1975b). Adipose linoleate levels should be periodically evaluated and vitamin E intakes appropriately adjusted (Witting and Lee 1975a).

Amount of adipose tissue as well as fatty acid composition has also been implicated in establishment of vitamin E requirement. Obese rats had threefold higher plasma α-tocopherol than normal weight rats Bieri and Evarts 1975a). On the other hand, obese rats had less α-tocopherol in lung and heart tissue than the control rats (Bieri and Farrell 1976).

Vitamin E intakes

While it is well established that dietary and tissue PUFA raise the vitamin E requirement and while there has been a continued trend in the U.S. to increase PUFA intake, there has been little concern over potential vitamin E deficiencies. Recent statistics indicate the average consumption of linoleic acid in the U.S. was 23g/person/day (Rizek et al. 1974). The average vitamin E intake from the diet was no more than 15 mg/person/day (Weiser and Salkeld 1977). In Australia a review of diets showed 7 out of 25 were inadequate in vitamin E according to established allowances (Nobile and Woodhill 1976). Examination of the dormitory food service meals at a Texas university revealed the E:PUFA ratio was less than 0.5, potentially deficient (Witting and Lee 1975b). Another estimation showed the average U.S. diet to have an E:PUFA ratio of 0.43 (Bieri and Evarts 1975b).

Failure to be concerned about these presumptively deficient vitamin E intakes rests on the comfortable assumption that increasing PUFA intake automatically increases tocopherol intake. This assumption has been challenged. Very little vitamin E occurs in fats of animal origin. The richest source is vegetable oil. Bieri and Evarts (1975b) concluded that all vegetable oils were satisfactory sources of vitamin E with such E:PUFA ratios as corn oil 0.49, soybean oil 0.60, and safflower oil 0.35. However, it was demonstrated that these ratios, as guides to adequacy, are useless except at low to moderate fat intakes. Furthermore, absorption of tocopherol may vary. Witting (1975) reported that increased ingestion of vitamin E resulted in decreased absorption. Weber et al. (1964) found that PUFA actually interfered with α-tocopherol absorption. Peake et al. (1972) were unable to confirm a differential absorption when feeding α-tocopherol with either corn oil or lard. More recent evidence showed fatty acids were absorbed more effectively than tocopherols (Weiser and Salkeld 1977). If this is true, then increasing dietary PUFA may be accompanied by increased dietary E, but actual appearance in circulation and body cells of excess PUFA does not necessarily mean concomitant appearance of more tocopherol. Moreover, much dietary vitamin E is the less potent γ-tocopherol. Even if the vitamin E:PUFA ratio of the diet were constant, the α-tocopherol: PUFA ratio of the absorbed matter and of the body tissues might be variable.

Functions of vitamin E

The functions of vitamin E in the organism are still debated. A 1976 review of vitamin E (Bieri and Farrell) proposed four roles of the vitamin: (1) As an antioxidant. There is extensive evidence tocopherols act in this capacity (Dillard et al. 1978b, Downey et al. 1978, Hafeman and Hoekstra 1977, Wilson et al. 1978). Vitamin E exerts its antioxidant capacity by disrupting the chain generation of free radicals in lipid peroxidation. That this is the sole or even major role of vitamin E is doubtful for other biological antioxidants exist, e.g., glutathione reductase, that appear to be more important in interrupting radical chain reactions. In addition, other antioxidants cannot prevent all derangements of vitamin E deficiency. (2) As a structural component of membranes. There is as yet no evidence to support this function, although Bonnetti and Novello (1976) reported that α-tocopherol was primarily localized within the cell bound to membranes. (3) As a regulator in protein synthesis. (4) As a regulator of enzyme activity. In vitamin E deficiency several enzymes do alter in activity, e.g., muscle creatine kinase, liver xanthine oxidase.

Effects of deficiency or excess

Although many fascinating effects of excessive or deficient dietary vitamin E have been observed, the succeeding paragraphs will consider only the six major effects that suggested the design and hypothesis of the present research. These are the effects on (1) hemolysis, (2) pulmonary response to oxygen stress, (3) tissue fatty acid patterns, (4) tissue tocopherol levels, (5) immunity, and (6) platelet aggregation.

The classic vitamin E deficiency test is degree of erythrocyte hemolysis in vitro. Although open to criticism as not nutrient-specific and insensitive to a range of vitamin E intakes, erythrocyte hemolysis is still frequently measured for correlation and comparison with other aspects of vitamin E nutriture. Yang and Desai (1977a) reported that vitamin E deficient rats exhibited spontaneous red blood cell (RBC) hemolysis in saline-phosphate buffer while vitamin E supplemented rats did not. Rats received a vitamin E deficient diet with 10% by weight molecular distilled corn oil as fat source. Rats receiving no vitamin E supplement had 87% hemolysis, while rats receiving 25-25,000 IU/kg diet/day dl-α-tocopheryl acetate had only 2% hemolysis. Although graded amounts of dl-α-tocopheryl acetate were fed, there was no difference in hemolysis once a critical minimum intake was achieved. As a measure of vitamin E nutriture, therefore, hemolysis was useful only in an either-or situation – either deficient or adequate – telling nothing about excessive intakes. A comparison of α- and γ-tocopherols confirmed that α-tocopherol protected against hemolysis at lower doses than γ-tocopherol (Aftergood and Alfin-Slater 1978). Horn et al. (1978) confirmed the relationship of vitamin E and hemolysis in Rhesus monkeys. Vitamin E was found to protect against oxidative damage to RBC membranes and hemoglobin in rats. Thiobarbituric (TBA) reactants were decreased, indicating inhibition of oxidation (Chow 1978).

Vitamin E appears to have a major protective role in lung. Premature infants frequently develop respiratory distress syndromes and are given respiratory assistance with enriched oxygen atmospheres. Prolonged treatment leads to severe toxic effects manifested in lung

degeneration and identified as bronchopulmonary dysplasia. Ehrenkranz et al. (1978) reported an attempt to forestall development of the disease by giving the infants intramuscular injections of vitamin E. None of the vitamin E treated infants developed bronchopulmonary dysplasia, while half of the control infants did. Vitamin E appeared to modify the toxic effects on lung of inspired high O_2 concentrations. This study has been sharply criticized (Northway 1978) for poor design, improper controls, and biased results. Nevertheless, the theory seems to be valid. Oxygen toxicity occurs in the lungs and is associated with increased lipid peroxidation (Dumelin et al. 1978). Vitamin E is known to antagonize lipid peroxidation (Dillard et al. 1978a). Tocopherol stores are low in infants, especially in premature infants (Goldbloom 1963, Wright et al. 1951). Animal studies support the hypothesis that vitamin E protects lung tissue from oxidative damage. When rats on diets either low or high in vitamin E for five weeks were exposed to ozone, the rats on the low vitamin E diet exhibited greater proliferation of mitochondria and greater O_2 consumption than the rats on the high vitamin E diet. This enhanced response to the ozone challenge with a low vitamin E diet suggested that vitamin E increased an animal's ability to withstand oxidant stress (Mustafa 1975). Chow (1977) confirmed these findings. Similar results have been reported in the mouse lung (Menzel et al. 1978). Vitamin E supplementation in the diet reduced oxidative damage in lung.

Dietary vitamin E influenced the total fatty acid content of plasma (Cho and Sugano 1978). Did tocopherol also influence the fatty acid pattern of tissue lipids? In vitamin E deficiency the levels of

C20:4ω6 rose in liver and gastrocnemius and quadriceps muscles of the rat (Bernhard et al. 1963, Witting and Horwitt 1967, Witting et al. 1967). Other investigators found that ω6 fatty acids, with the exception of C20:4ω6 rose in phospholipids of vitamin E deficient rats (Lee and Barnes 1969). Further attempts to clarify this relationship sometimes failed to demonstrate an effect by vitamin E on FA composition of tissues. Using chicks, Miller and White (1975) explored possible effects of three different antioxidants on tissue fatty acid patterns. Neither selenium, nor dl-α-tocopherol, nor ethoxyquin had any influence on the distribution of fatty acids. Donovan et al. (1977) fed rats 5% stripped lard or 5% stripped corn oil, supplemented with 0, 10.5, or 105 mg/kg dl-α-tocopheryl acetate. All diets were EFA sufficient and supplemented with sodium selenate. After three weeks diet and lung fatty acids were equilibrated. Although the source of dietary fat altered relative concentrations of pulmonary fatty acids, dl-α-tocopheryl acetate supplementation had no effect. Recent work by these same authors duplicated the results in another species. Vitamin E, whether supplemented at 0, 10.5, or 105 mg/kg diet, had no significant effect on mouse lung fatty acid composition (Donovan and Menzel 1979). Observations of the fatty acid patterns of platelets established lack of a relationship between the levels of α-tocopherol and individual FAs (Schoene and Lehmann 1978). A rise in C20:4ω6 and a decline in C18:2ω6 on a vitamin E deficient diet was apparent in rabbit muscle but less obvious in heart, liver, lung, or kidney (Chan et al. 1978). A possible explanation for these inconsistencies came from research by Farnsworth et al. (1979). They studied the effects of vitamin E and selenium

deficiencies on the fatty acid composition of rat retinal tissues. A dietary deficiency of either antioxidant caused a large decrease in the total PUFA of rat retinal pigment epithelium with slight increases in C18:2ω6 and C20:4ω6. In contrast, there were no changes in the whole retina. Thus, fatty acid compositional alterations were not generalized to all tissues when changing antioxidant intakes.

In contrast to fatty acids, tissue tocopherol levels were more clearly related to dietary tocopherol. Lehmann et al. (1977a) fed human volunteers 7.6 mg α-tocopherol/day for 40 days followed by 12.9 mg α-tocopherol/day for 40 days. In both females and males the plasma tocopherol levels rose in response to the dietary increase. However, the dietary fat also increased and plasma total lipids increased which has been shown to influence plasma tocopherol. Realizing that assessment of vitamin E status using plasma is complicated by plasma total lipid levels, Lehmann (1978) then measured only platelet tocopherol. She reported a logarithmic relationship between dietary and platelet tocopherol and no relation between platelet tocopherol and plasma total lipids. Thus, this method was proposed as superior for assessment of vitamin E nutriture. Yang and Desai (1977b) showed a definite hepatic deposition of α-tocopherol in response to increasing dietary tocopherol intakes. In contrast, pulmonary tocopherol increased only slightly when the tocopherol content of the diet increased (Aftergood and Alfin-Slater 1978).

Vitamin E appears linked to immunological functions. In the mouse vitamin E supported β-cell mitogenesis in spleen and lymph node (Shloss and Corwin 1977). In adult male rats fed 500 mg dl-α-tocopheryl acetate

three times weekly, the ability of mixed leukocytes to kill Staphylococcus aureus was totally eliminated. Excess vitamin E appeared to inhibit the oxygen-dependent free radical process involved in the bactericidal activity of leukocytes (Fong et al. 1978). Authors in India giving adult and young human males 300 mg dl-α-tocopheryl acetate/day for three weeks also found significantly depressed bactericidal activity by leukocytes and a depression in mitogen-induced lymphocyte transformation. A simultaneous in vivo test of skin hypersensitivity showed vitamin E had no effect. At the same time, one subject on vitamin E experienced complete relief of a nasal allergy and another had improvement of asthmatic attacks (Prasad 1980). Thus, vitamin E had differential effects on different limbs of immune response. Corwin and Shloss (1979) reported vitamin E to be a mitogen acting on the immune response by a mechanism other than antioxidation. At suboptimal and at high levels vitamin E was more effective as a stimulant to the thymus-dependent lymphocyte response to conconavalin A than vitamin E at normal dose levels (Corwin and Shloss 1980).

The connection between tocopherol and immunity may be through prostaglandins. Chickens fed a high vitamin E diet exhibited reduced mortality from an E. coli challenge as well as depressed PGE_1, PGE_2, and $PGF_{2\alpha}$ in spleen and in bursa (Likoff et al. 1978). This suggested vitamin E enhanced the immune response by reducing PG production.

Dietary vitamin E has been implicated in irregularities of platelet aggregation. A PUFA-rich vitamin E-deficient diet fed to pigs was associated with pronounced platelet thrombosis (Nafstad 1974). Coagulation could be induced in rats on either an α-tocopherol deficient diet

or a lipid rich diet. However, if the high lipid diet were supplemented with α-tocopherol, the animals were protected (Fong 1976). Corrigan and Marcus (1974) reported a case study of a patient with delayed coagulation time who had been self-administering excessive doses of vitamin E. A 16-month-old infant with signs of vitamin E deficiency had abnormal aggregation (Khurshid et al. 1975). Platelet aggregation occurred with a biphasic response — the immediate phase and the second wave. Addition of α-tocopherol to the incubation medium did not affect the first phase but inhibited the second. This second phase could also be inhibited by indomethacin and acetylsalicylate sodium (Fong 1976). Not only α-tocopherol but its nicotinate and acetate esters exerted inhibitory effects on arachidonate-induced platelet aggregation (Svensson and Oki 1978). As platelets aggregated, lipid peroxides were released. When α-tocopherol was added to the incubation medium at concentrations up to 1.5 mM, a dose-dependent reduction in platelet aggregation was noted. There was rapid uptake of α-tocopherol by platelets, and a linear relation was established between platelet and concentration of α-tocopherol in the incubation medium. Alpha-tocopherol inhibited the second wave of aggregation and decreased lipid peroxide release from the platelets (Steiner and Anastasi 1976). However, in vitro incubation of platelets with vitamin E failed to inhibit the platelet lipoxygenase or depress ^{14}C-HETE synthesis from ^{14}C-arachidonate (Gwebu et al. 1978).

Vitamin E and PG synthesis

The influence of vitamin E on immunity, lung protection, and platelet aggregation suggests a physiological role in PG metabolism. Many studies have hinted that PG synthesis is altered in vivo by vitamin E, though few actual measures have been taken. Lands et al. (1973) found that 500 μM α-tocopherol inhibited PG synthesis by 50% and concluded that the inhibition was related to the structure of the enzyme (sheep vesicular gland oxygenase) and not the antioxidant ability alone. Although α-tocopherol added to the incubation medium significantly inhibited soybean lipoxygenase, it had no effect on mammalian cyclooxygenase as measured by incorporation of label in product in a bovine vesicular gland microsomal system (Panganamala et al. 1977). Vitamin E at 1.0 mM to 5.0 mM failed to inhibit PG synthesis (Zenzer and Davis 1978). Thus, results of the in vitro work are contradictory.

Nearly all the in vivo investigations of the effect of vitamin E on PG synthesis have been conducted by one laboratory and have focused on vitamin E deficiency. Feeding a vitamin E deficient diet of 7% stripped lard and 3% stripped cod liver oil to rabbits, Chan et al. (1978) reported a metabolite of $PGF_{2\alpha}$ in plasma was higher in deficient than in control animals. Later, using a microsomal preparation from rabbit semitendinosus muscle, they found a significant depression in PGE_2 and $PGF_{2\alpha}$ which was restored to normal after supplementation with oral tocopherol (Chan et al. 1979). This depressed synthesis of prostaglandins, they felt, explained in part the observed elevations in muscle C20:4ω6 in vitamin E deficiency. Skeletal muscle cyclooxygenase normally produced PGE_2 and $PGF_{2\alpha}$ in a 1:1 ratio. While vitamin E deficiency

significantly reduced the enzyme activity, the $PGE_2/PGF_{2\alpha}$ ratio was unchanged. Cyclooxygenase activity returned to normal within 48 hours of oral tocopherol supplementation (Chan et al. 1980a). The key enzyme controlling PG catabolism is 15-hydroxy-prostaglandin dehydrogenase (PGDH). Vitamin E deficiency elevated activity of PGDH in skeletal muscles of rabbit but not in heart or kidney (Chan et al. 1980b). Prostaglandin synthesis was also depressed in testis during vitamin E deficiency (Thuy et al. 1978). In contrast, Hope et al. (1975) reported PGE_1, PGE_2, and $PGF_{2\alpha}$ were elevated in serum from vitamin E deficient animals.

Little work has been done on the effects of excessive vitamin E on PG synthesis in vivo. Likoff et al. (1978) measured PGs in chickens which received normal and high vitamin E diets. Splenic PGE_1, PGE_2, and $PGF_{2\alpha}$ synthesis was inhibited by the high vitamin E diet. Hope et al. (1975) fed rats four different levels of α-tocopherol and measured serum PGs. There was an inverse dose-response. The higher the serum tocopherol level, the lower the level of PGE_2 and $PGF_{2\alpha}$.

The evidence, both direct and indirect, suggests a relationship between vitamin E and PG synthesis. It remains to clarify the relationship, the effective doses and isomers, the responsive prostanoids, the mechanism, and the functional significance.

METHODS

Experimental Design

Animals in Experiment I were randomly assigned to control or EFAD groups. In Experiment II seventeen litters of four or five mates for a total of 80 rats were depleted of EFA for 45 days. Results from the first experiment showed that a 45-day depletion period would provide a population whose triene:tetraene ratio was > 0.4. Previous experience also indicated that a few animals would fail to grow uniformly or would develop diseases, or otherwise become unfit for continuance in an experiment. Therefore, from the 80 EFAD animals we were able to select a uniform population of 60 animals, 4 littermates from each of 15 litters for application of the experimental treatments. In Experiment III 60 rats were depleted of EFA for 45 days. From this population 40 animals were selected for continuation in the experiment.

On the 46th experimental day animals were randomly assigned to one of the six experimental groups in Experiment II or four experimental groups in Experiment III. Animals receiving 0, 1, 5, 10, 20, or 50 mg vitamin E supplementation/day were designated 0-T, 1-T, 5-T, 10-T, 20-T, or 50-T, respectively. Figures 3 and 4 detail the experimental design. Animals assigned to EFAD groups were killed on the 46th experimental day. Animals in groups 0-T through 50-T were lightly anesthetized with ether. A blood sample was taken from the orbital sinus. Each animal was then weighed and changed to a 20% safflower oil diet (SO) and to one of the dl-α-tocopheryl acetate supplements. Animals in groups 0-T through 50-T received the experimental treatment until the 91st experimental day, at which time they were sacrificed.

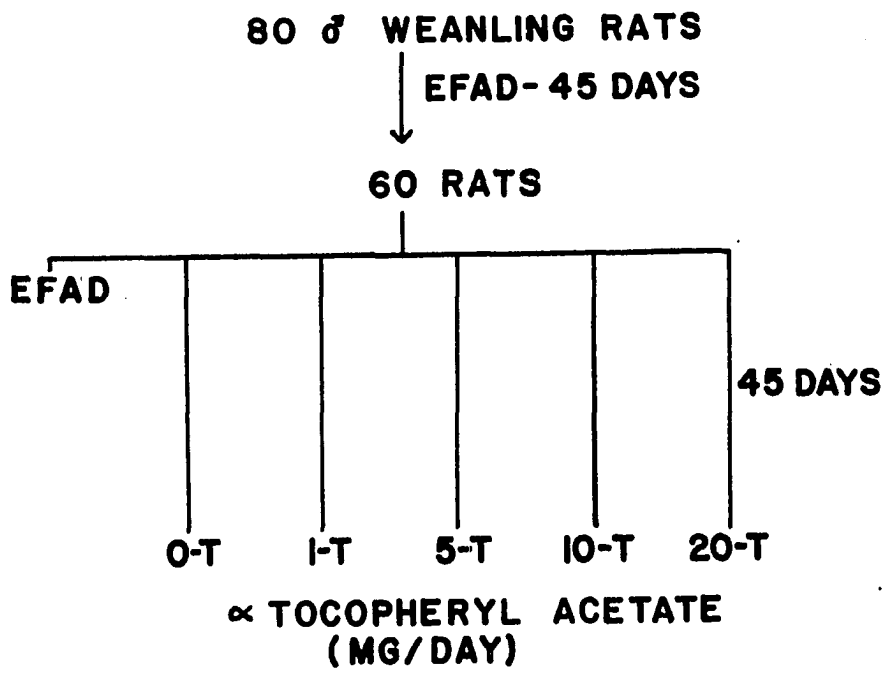

Figure 3. Design of Experiment II

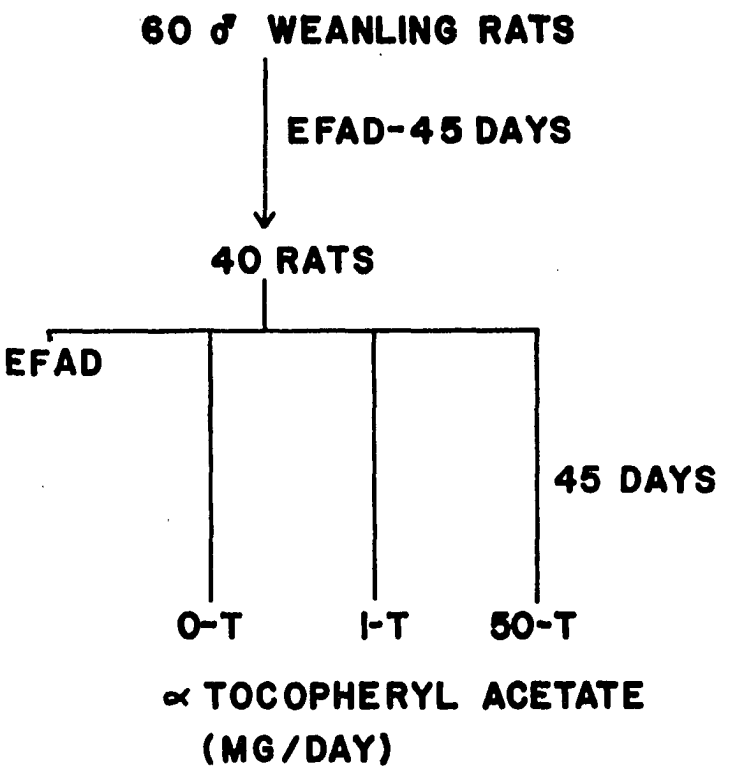

Figure 4. Design of Experiment III

Purposes of Experiments

Experiment I was undertaken to establish the length of time required to deplete body stores of EFA to a target point of serum T/T $\geq$ 0.4. We wished animals to remain in good health and to show only the commencement of EFA deficiency. Experiment II was designed to observe the effect of graded levels of α-tocopherol supplementation on growth, body weight, food efficiency, erythrocyte hemolysis, serum and pulmonary FA composition, and pulmonary α-tocopherol stores. The purpose of Experiment III was to replicate the results of Experiment II on selected dependent variables, to provide a further increase in dose of α-tocopherol to provoke a response, and to assay PG synthetic rate in lung and serum.

Treatment of Animals

All animals were weanling male Wistar rats from the stock colony of the Department of Food and Nutrition, Iowa State University. The animals were housed in individual 1/2-inch wire mesh cages in a room maintained at approximately 25^{o} and 45-55% relative humidity. In Experiment I the animals experienced the following light-dark cycle: 1100-2300 hours dark, 2300-1100 hours light. In Experiments II and III the photoperiod was shifted slightly so the animals experienced light from 0100 to 1300 hours and darkness from 1300 to 0100 hours daily. These hours were selected to permit the animals entrance into the dark cycle, their active time, just after feeding and watering activities were completed. In this way the animals experienced the least

disruption of their resting phase and had fresh food and vitamins available at the onset of their active phase. Animals were provided fresh food every other day, vitamin supplements daily, and clean cages and water bottles weekly. Water-soluble and fat-soluble vitamin supplements were given in small glass dishes. After a few days animals had learned to consume these quantitatively within a few minutes following administration. Food and distilled water were available ad libitum. The animals were weighed every other day. Food intake was determined every other day by collecting all spilled food and weighing it along with the uneaten food remaining in the diet jar.

Diets and Supplements

Composition of the essential fatty acid deficient (EFAD), control, and 20% safflower oil (SO) diets appears in Table 2.

The water-soluble vitamin supplement was used throughout the course of all the experiments. All animals in all groups received 1.0 ml of the water-soluble vitamin supplement each day. Composition appears in Table 3.

Fat-soluble supplements were measured into vitamin cups with calibrated droppers. Fat-soluble vitamin supplements were prepared by mixing weighed amounts of each vitamin in a small amount of appropriate oil. Oil was added to bring each supplement up to the weight predetermined to provide proper daily dosage by the calibrated dropper. Tables 4, 5, and 6 show the fat-soluble vitamin supplements used in Experiment I, Experiment II, and Experiment III, respectively. To

Table 2. Composition of essential fatty acid deficient (EFAD), control, and 20% safflower oil (SO) diets

Ingredient	% by weight		
	EFAD	Control	SO
Casein, vitamin free, test[a]	26.0	26.0	26.0
dl-methionine[b]	0.39	0.39	0.39
Williams-Briggs modified mineral mix[c]	3.5	3.5	3.5
Nonnutritive fiber[d]	2.0	2.0	2.0
Hydrogenated coconut oil[a]	20.0	18.0	–
Corn oil[e]	–	2.0	–
Safflower oil[f]	–	–	20.0
Cornstarch[g]	48.11	48.11	48.11

[a]U.S. Biochemical Corp., Cleveland, Ohio.

[b]Grand Island Biological Co., Grand Island, New York.

[c]Williams et al. (1968), Teklad Test Diets, Madison, Wisconsin.

[d]Teklad Test Diets, Madison, Wisconsin.

[e]Mazola, Best Foods, CPC International, Englewood Cliffs, New Jersey.

[f]Pacific Vegetable Oils International, Richmond, California.

[g]Clinton Corn Processing, Clinton, Iowa.

Table 3. Composition of water-soluble vitamin supplement

Ingredient	Dose/rat/day
Thiamine HCl[a]	29 μg
Riboflavin[a]	39 μg
Pyridoxine HCl[a]	20 μg
Folic acid[a]	20 μg
D-Ca-pantothenate[b]	97 μg
p-amino-benzoic acid[a]	97 μg
Vitamin B_{12}[a]	0.2 μg
Biotin[c]	2.0 μg
Niacin[a]	64 μg
i-Inositol[a]	2.4 mg
Choline Cl[c]	4.8 mg
20% ethanol	1.0 ml

[a]Grand Island Biological Co., Grand Island, New York.

[b]ICN Pharmaceuticals, Inc., Cleveland, Ohio.

[c]General Biochemicals, Inc., Chagrin Falls, Ohio (known as Teklad, Madison, Wisconsin, since 1975).

Table 4. Composition of fat-soluble vitamin supplement for Experiment I and for all EFAD groups

Ingredient	Dose/rat/day
Vitamin K_1[a]	10 μg
dl-α-tocopheryl acetate[b]	1.0 mg
Retinyl palmitate[c]	17 μg
Vitamin D_3[c]	0.6 μg
Tricaprylin[d]	34-44 mg[e]

[a]Sigma Chemical Co., St. Louis, Missouri.

[b]Teklad Test Diets, Madison, Wisconsin.

[c]Grand Island Biological Co., Grand Island, New York.

[d]Nutritional Biochemicals, Cleveland, Ohio.

[e]Weight appropriate to calibration of dropper.

avoid administering EFA, we used tricaprylin as carrier for fat-soluble vitamins in EFAD.

Food Efficiency Ratios

Food efficiency ratios were calculated by dividing food intake for the period by weight gained during the period.

$$FER = \frac{\text{food intake (g)}}{\text{weight gain (g)}}$$

Table 5. Composition of fat-soluble vitamin supplements for Experiment II

Ingredient	Dose/rat/day				
	0-T	1-T	5-T	10-T	20-T
Vitamin K_1[a]	10 μg	10 μg	10 μg	10 μg	10 μg
dl-α-tocopheryl acetate[b]	–	1.0 mg	5.0 mg	10.0 mg	20.0 mg
Retinyl palmitate[a]	17 μg	17 μg	17 μg	17 μg	17 μg
Vitamin D_3[a]	0.6 μg	0.6 μg	0.6 μg	0.6 μg	0.6 μg
Corn oil[c]	40 mg[d]	39 mg[d]	35 mg[d]	40 mg[d]	30 mg[d]

[a]Grand Island Biological Co., Grand Island, New York.

[b]Teklad Test Diets, Madison, Wisconsin.

[c]Mazola, Best Foods, CPC International, Englewood Cliffs, New Jersey.

[d]Weight appropriate to calibrated dropper used.

Nonterminal Blood Sampling

In Experiment I blood samples were taken from four control animals and 11 EFAD animals on experimental day 22. Blood samples were taken from groups 0-T through 50-T in Experiments II and III on experimental day 46. Each animal was lightly anesthetized with ether. A 100 μl glass capillary tube was carefully inserted behind the right eye into the orbital sinus, and an average of 1.2 ml whole blood was drawn into a graduated glass centrifuge tube chilled in an ice bath. Bleeding was

Table 6. Composition of fat-soluble vitamin supplements for Experiment III

Ingredient	Dose/rat/day		
	0-T	1-T	50-T
Vitamin K_1[a]	10 μg	10 μg	10 μg
dl-α-tocopheryl acetate[b]	–	1.0 mg	50.0 mg
Retinyl palmitate[a]	17 μg	17 μg	17 μg
Vitamin D_3[a]	0.6 μg	0.6 μg	0.6 μg
Corn oil[c]	55 mg[d]	64 mg[d]	100 mg[d]

[a]Grand Island Biological Co., Grand Island, New York.

[b]Teklad Test Diets, Madison, Wisconsin.

[c]Mazola, Best Foods, CPC International, Englewood Cliffs, New Jersey.

[d]Weight appropriate to calibrated dropper used.

stopped at once by applying pressure to a cool moist cottonball over the eye. Blood was centifuged at 600 x g for 20 minutes at 4°. Serum was transferred to one-dram vials, flushed with nitrogen, and stored at -20° awaiting further analysis.

Necropsy

All the animals of Experiment I and all EFAD animals of Experiments II and III were killed on experimental day 46. The remaining rats were killed on experimental day 91. All were allowed to eat until

sacrifice by cervical dislocation[1].

Blood was taken immediately by heart puncture in the left ventricle.

In Experiment I all the blood was placed in covered centrifuge tubes in an ice bath, and held no more than 30 minutes before further processing. Whole blood was centrifuged at 600 x g for 20 minutes at 4°. The serum was transferred to one-dram vials, layered with nitrogen, and stored at -20° until further analysis. In Experiment II one drop whole blood was added to 3.0 ml saline-phosphate buffer at room temperature for hemolysis determination and was held at room temperature no longer than 30 minutes before continuing the hemolysis analysis. The remaining blood was handled as in Experiment I. In Experiment III one ml of blood was placed in a cooled glass centrifuge tube and treated as in Experiment I. The remaining blood was immediately incubated at 37° for 10 minutes for PG analysis. At the end of incubation a 0.1 volume of 4.2 mM solution of aspirin in KPi buffer (pH 7.4) was added and the tube cooled in ice and centrifuged at 600 x g for 20 minutes at 4°. Serum was transferred to a one-dram vial and stored at -20°.

In all three experiments lungs were excised, weighed, immediately frozen in liquid nitrogen, sealed in plastic pouches, and stored at -20° until further analysis. In Experiment III the left lung was handled separately. Immediately upon removal the left lung was weighed, and homogenized in 5.0 ml cold KPi buffer with a Brinkman[2] polytron PCU-2-110 at rheostat setting 5.5 for 60 seconds. The homogenate was incubated in

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[1]Cervical Dislocators, Inc., Wausau, Wisconsin.

[2]Brinkman Instruments, Westbury, New York.

a shaking water bath at 37° for ten minutes. At the end of incubation 2.5 ml of 42 mM aspirin in KPi buffer (pH 7.4) was added and the mixture stored at -20°.

Chemical Analyses

Lipid extraction

Total serum lipid was extracted by the method of Sperry and Brand (1955), using 0.5 ml serum. The resultant extract was made up to a 5.0 ml volume in dry chloroform and stored under N_2 at -20°. Total lung lipids were extracted by the method of Folch *et al*. (1957), brought up to a 5.0 ml volume in dry chloroform, and stored under N_2 at -20°.

Methylation

Both serum and lung lipid extracts were methylated by placing a 2.0 ml aliquot of the extract in a screw-capped culture tube. Extract was evaporated to dryness at room temperature under a stream of nitrogen. Immediately 1.0 ml benzene, 1.0 ml methanol, and 1.0 ml 14% BF_3-methanol[1] were added. Contents were mixed by shaking briefly. Tubes were flushed with nitrogen and closed tightly with teflon-lined screw caps. Tube contents were heated in a boiling water bath for 30 minutes. Methyl esters were extracted by adding two volumes (4 ml) hexane and one volume (2 ml) distilled water. Tube contents were thoroughly mixed for ten seconds, then centrifuged 10 minutes at 1100 x g until layers were clearly

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[1]Applied Science Laboratories, State College, Pennsylvania.

separated. The top layer was transferred to a 2-dram vial, flushed with nitrogen, capped, and stored at -20°.

Gas chromatography

The resultant fatty acid methyl esters were concentrated under nitrogen and warmed to room temperature before a 1.0 µl aliquot was injected into a Beckman[1] GC 72-5 flame ionization gas chromatograph equipped with two columns. Peak areas were determined by an Infotronics[2] Automatic Digital Integrator Model CRS-208 on one column and a Columbia Scientific Industries[3] Supergrator-1 Computing Integrator on the other. The columns themselves were 6-foot, stainless steel, 1/8-inch diameter, packed with W-AW support, 100-120 mesh, and 10% cyano silicone-10 liquid phase[4]. Conditions of operation were as follows: columns 180°, detector 250°, inlets 55°, line 55°, nitrogen (carrier gas) flow rate 20 ml/min, make-up gas flow rate 60 ml/min, H_2 flow rate 45 ml/min, air flow rate 300 ml/min, full-scale amps = 1×10^{-10}. Correction factors were developed using standards of known composition. Fatty acids were identified by comparison of their retention times with those of known standards[5].

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[1]Beckman Instruments, Inc., Fullerton, California.

[2]Infotronics Corp., Austin, Texas.

[3]Columbia Scientific Industries, Dallas, Texas.

[4]Alltech Associates, Arlington Heights, Illinois.

[5]Applied Science Laboratories, State College, Pennsylvania.

Hemolysis

The method for determining hemolysis was that of Draper and Csallany (1969). The only modification in the method was a shortening of the incubation time from four to three-and-a-half hours. We found that precisely the same results could be obtained with the shorter incubation time. Absorbance of the sample was determined on a Beckman[1] DU Spectrophotometer, Model 2400, equipped with an Update Instrument[2] Digital Display Readout.

Alpha-tocopherol

The α-tocopherol content of lung was determined by a modification of Bieri's (1968) procedure. The frozen lung was rapidly minced with stainless steel scissors and a randomized sample of approximately 0.5 g was placed in a screw-capped glass test tube containing 1.5 ml 8% ascorbic acid in 5 mM Na_2EDTA, and 1.0 ml redistilled ethanol. From this point on, all procedures were performed in a darkened room, the only light coming from the hallway through a frosted glass panel in the door. The tube contents were layered with nitrogen, capped, and placed in a 65° water bath. After five minutes 1.0 ml saturated KOH solution (made by adding 15 g KOH to 11 ml deionized, distilled water) was added. The tube contents were layered with nitrogen, capped, and placed in the 65° water bath for 20 additional minutes. Several times during digestion and saponification, the tube was briefly shaken. At the end of 20 minutes, the tissue fully digested, the tube was placed in an ice bath. When

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[1]Beckman Instruments, Inc., Fullerton, California.

[2]Update Instruments, Madison, Wisconsin.

cooled, the saponified mixture was extracted with 4.0 ml redistilled hexane three times for a pooled extract total of 12 ml. Three ml aliquots were transferred in duplicate to 15 ml ground glass-stoppered centrifuge tubes and evaporated to dryness under nitrogen at room temperature. The walls of the tube were washed down with progressively smaller volumes of benzene three times until all product was concentrated in the tip of the tube. This concentrate was quantitatively applied under a stream of nitrogen to a 20 cm by 20 cm glass plate. The plate was coated with a silica gel-sodium fluorescein solution of 250 microns thickness which was prepared by rapid mixing of 30 g silica gel G[1] and 60 ml of 0.004% sodium fluorescein in distilled water. Plates were air dried one hour, placed in a 100° oven for two hours, and stored in a dessicator cabinet for use within two days. Immediately upon sample application, the plate was placed in a solvent tank containing benzene: ethanol (150:3 v/v) for about 60 minutes. Upon removal, the plate was flushed with nitrogen, the α-tocopherol spot located by ultraviolet lamp, and the spot immediately scraped into a centrifuge tube. Two ml of freshly prepared 0.02% bathophenanthroline[2] in redistilled ethanol were added and the tube contents vigorously mixed 20 seconds. The tube was centrifuged two minutes at 1100 x g and the supernatant decanted. The silica gel was eluted a second time and eluates pooled. All procedures were performed under nitrogen, in the dark, as rapidly as possible to prevent α-tocopherol oxidation. A two ml aliquot of eluate was

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[1]Brinkman Instruments, Westbury, New York.

[2]G. Frederick Smith Chemical Co., Columbus, Ohio.

placed in a cuvette and 200 µl freshly prepared 0.03% $FeCl_3$ in redistilled ethanol added. Absorbance of the reaction mixture, briefly stirred, was read at 534 nm after two minutes. All samples were analyzed on a Beckman[1] DU Spectrophotometer, Model 2400, equipped with an Update[2] Instrument Digital Display Readout. Alpha-tocopherol content of the cuvette was determined by reference to a standard curve, and α-tocopherol content of the tissue was calculated.

Measurement of prostaglandins

The level of prostaglandins present in serum and in lung homogenate after a ten-minute incubation period was determined by radioimmunoassay as described by McCosh et al. (1976). The specific antisera, normal rabbit serum (NRS), and anti-rabbit gamma globulin (ARGG) used in the assays are described in the Appendix (Table 27). Compositions of the three buffers used appear in the Appendix (Tables 28, 29, and 30).

Range-finding experiments demonstrated the dilutions necessary to place the tissue and serum samples within the range of sensitivity of the assay. Accordingly, all serum samples were diluted 1:4 in PBS gel and pulmonary homogenates were diluted 1:100 or 1:10 in PBS gel.

On the first day of each assay the appropriate antiserum (anti-PGE_1, anti-PGE_2, etc.) was diluted with normal rabbit serum to a predetermined, appropriate titer for the assay. Working dilutions of ARGG and NRS were mixed in a 1:1 ratio while the diluted antiserum and ARGG were also mixed in a 1:1 ratio. Both solutions were then allowed to

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[1]Beckman Instruments, Inc., Fullerton, California.

[2]Update Instruments, Madison, Wisconsin.

pre-precipitate overnight (or 6-24 hours) at 4°. Appropriate dilutions of reagents varied and were determined for each batch.

After the pre-precipitation incubation period each diluted sample was assayed in duplicate and two standard curves were determined concurrently with each assay set. Tubes were included in each assay to determine total counts/tube, background binding by NRS, and binding capacity of the antiserum. Assays were always conducted over ice and all reagents maintained at 4°. Each assay tube was prepared with sequential additions of PBS-gel, sample or standard, antiserum, and ^{3}H-labelled prostaglandin. A representative protocol appears in the Appendix, Table 31. The tubes were shaken gently, wrapped tightly with parafilm, placed in a closed plastic bag and incubated at 4° for 12-36 hours. After incubation each tube received 3.0 ml cold PBS and was centrifuged immediately at 8° at 2000 x g for 30 minutes. Supernatant was poured off and discarded. To the pellet was added 0.3 ml deionized, distilled water and 3.5 ml liquid scintillation counting cocktail[1]. Each tube was capped, briefly mixed and counted in a Packard[2] Tri-Carb Liquid Scintillation Spectrometer, C2425, in the ^{3}H mode, for either 10 minutes or 10,000 cpm, giving an error of usually 2.0% or less. The data were analyzed by the radioimmunoassay computer program designed by Duddleson et al. (1972) to provide concentrations per ml original homogenate or serum for each sample. Corrections for cross reactivity were made using simultaneous equations as proposed by McCosh et al. (1976). The fractional

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[1]Scintiverse, Fisher Scientific Co., Fairlawn, New Jersey.

[2]Packard Instrument Co., Downer's Grove, Illinois.

binding of PGE_2 with PGE_1-antiserum was 0.20 and PGE_1 with PGE_2-antiserum was 0.17.

Statistical Analyses

Treatment means, standard error of the means, t tests, analysis of variance, and linear regression were calculated via SAS computer programming. In Experiment II a randomized balanced incomplete block design was employed as described by Cochran and Cox (1957). See Appendix, Table 32. In Experiment III animals were placed in treatment groups according to a randomized complete block design. In both cases by analysis of variance assuming a linear model, we were able to separate genetic effects from treatment effects. A standard of $p < .05$ was applied to identify significant differences among groups. In Experiment II analysis of variance using both five and six group comparisons were made. In Experiment III analysis of variance using both three and four group comparisons were made. The six and four group comparisons permitted analysis of the effect of EFAD on dependent variables. The five and three group comparisons removed the effect of age differences and allowed straight comparison of α-tocopherol affects.

RESULTS

Analysis of variance (ANOVA) was performed on all dependent variables in Experiments II and III. Two types of analyses were run — one including and one excluding the EFAD group. Inclusion of the EFAD group allowed determination of the effect of fat source (FS) on dependent variables. Exclusion of the EFAD group permitted determination of the effect of α-tocopheryl acetate (T) on dependent variables. Where ANOVA indicated possible group differences, t-tests were conducted to isolate the specific groups affected.

Feeding graded levels of dl-α-tocopheryl acetate with 20% safflower oil resulted in few statistically significant differences among groups. In the following presentation of results, therefore, differences will be indicated only when statistically significant or suggestive.

Experiment I

Body weight and food intake

Mean body weights were calculated at two time points during the experiment — after 19 and after 45 days on the diets. After 19 days the EFAD group weighed 135 ± 3g and the control 139 ± 9g. After 45 days the EFAD group averaged 301 ± 8g and the control group 322 ± 11g (Table 7). The EFAD group weighed slightly, but not significantly, less.

Weight gains over the first 19 days of experimental feeding were 87 ± 8g for the control group and 84 ± 3g for the EFAD group. Weight gains for the succeeding period (experimental days 20 to 46) were 183 ± 3g

Table 7. Body weights, weight gains, food intakes and food efficiency ratios. Experiment I

	Control (n = 4)	EFAD (n = 11)
Body weight (g)		
19 day	139 ± 9[a]	135 ± 3
46 day	322 ± 11	301 ± 8
Weight gain (g)		
Days 1-19	87 ± 8	84 ± 3
Days 20-46	183 ± 3	166 ± 7
Total food intake (g)	537 ± 19	517 ± 13
FER	0.50 ± 0.01	0.48 ± 0.03

[a]Mean ± SEM.

for the control group and 166 ± 7g for the EFAD group (Table 7).

Total food intake for the control group was 537 ± 19g and for the EFAD group 517 ± 13g. Calculated food efficiency ratios were 0.50 ± 0.01 and 0.48 ± 0.03 for control and EFAD groups, respectively (Table 7).

Serum fatty acid profile

Fatty acid patterns were determined on serum samples taken at two time points during the experiment in order to monitor progress of the development of EFA deficiency.

After 21 days sharp differences were beginning to appear between EFAD and control animals. On the EFAD diet serum palmitate, palmitoleate, stearate, oleate, and eicosatrienoate rose while linoleate and arachidonate declined. The saturated and monounsaturated fatty acids and their metabolic products became more pronounced as the levels of essential fatty acids dropped (Figure 5). Palmitate levels in control and EFAD

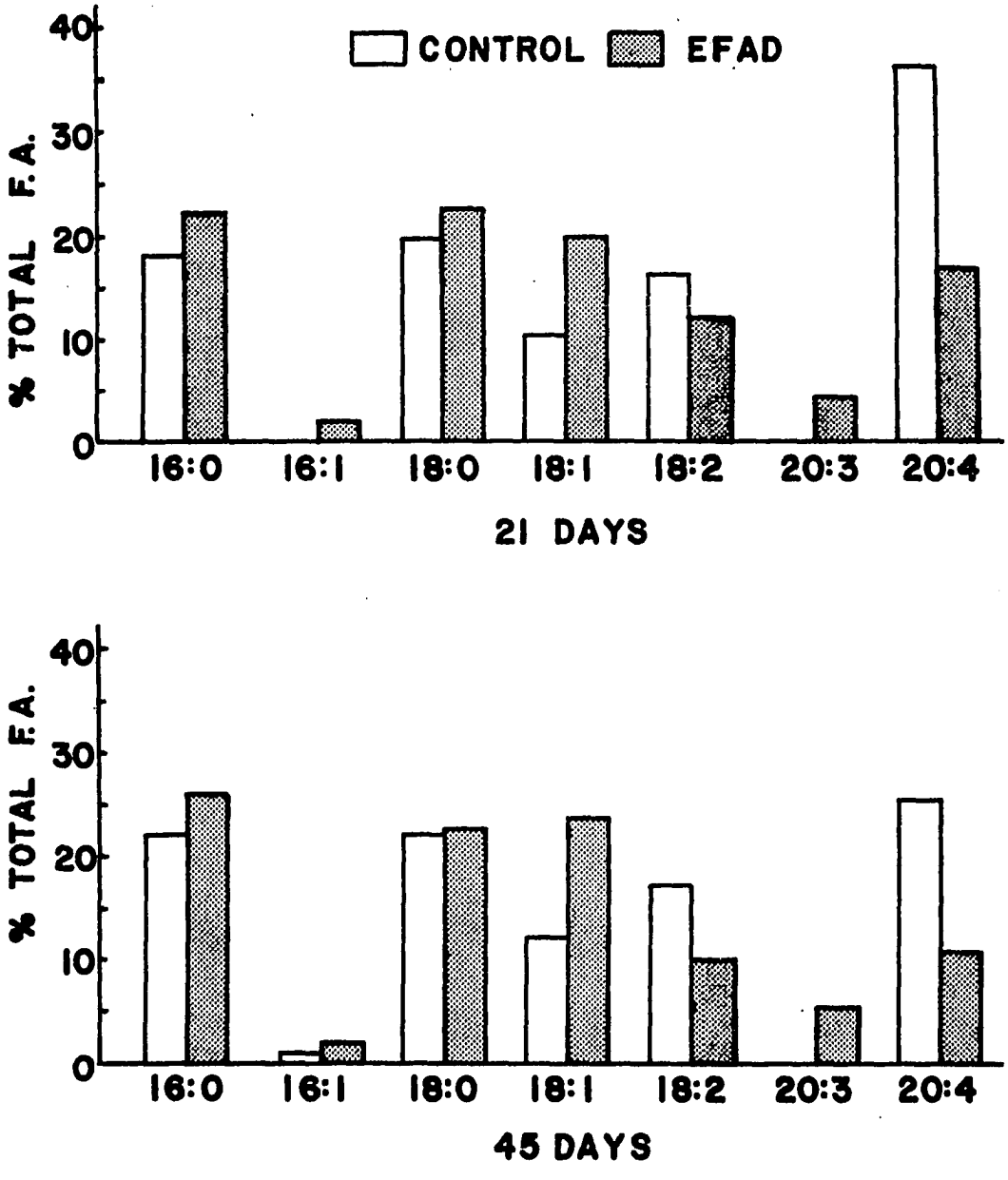

Figure 5. Serum fatty acid patterns after 21 and 45 days of feeding control or essential fatty acid deficient diets. Experiment I

groups were 18.22 ± 0.38% and 22.44 ± 0.73% ($p < .05$). The differences between the two groups in level of stearate, oleate, linoleate and arachidonate were significant at $p < .01$. Oleate in control and EFAD groups was 10.19 ± 0.38% and 20.02 ± 0.36%, respectively. Arachidonate after only 21 days of EFA deficient diet was half of control levels, 16.71 ± 1.09% vs 35.75 ± 3.57%. Eicosatrienoate, undetectable in the control group, was 4.42 ± 0.80% in the EFAD group ($p < .05$, Table 8).

Table 8. Serum fatty acid patterns after 21 and 45 days of feeding control or essential fatty acid deficient diets. Experiment I

	% of total fatty acids measured			
	21 days		45 days	
Fatty acid	Control	EFAD	Control	EFAD
16:0	18.22 ± 0.38[a]	22.44 ± 0.73*	22.07 ± 0.44	26.14 ± 0.85*
16:1	0	1.72 ± 0.30*	1.25 ± 0.24	1.93 ± 0.49
18:0	19.53 ± 0.62	22.59 ± 0.45**	22.18 ± 0.46	22.51 ± 0.78
18:1	10.19 ± 0.38	20.02 ± 0.36**	12.17 ± 0.75	23.52 ± 0.73**
18:2ω6	16.32 ± 2.20	12.08 ± 0.50**	17.01 ± 0.31	10.08 ± 0.69**
20:3ω9	0	4.42 ± 0.80*	0	5.38 ± 0.30**
20:4ω6	35.75 ± 3.57	16.71 ± 1.09**	25.32 ± 0.60	10.45 ± 0.73**
T/T	0	0.26	0	0.51

[a]Mean ± SEM.

*t, $p < .05$.

**t, $p < .01$.

After 45 days a similar pattern with minor differences appeared. Palmitate, oleate, and eicosatrienoate were significantly higher in the EFAD animals than in controls, and linoleate and arachidonate levels were depressed. Linoleate level was 17.01 $\pm$ 0.31% in the control group and 10.08 $\pm$ 0.69% in the EFAD group ($p < .01$). The level of arachidonate in the control vs EFAD animals was 25.32 $\pm$ 0.60% vs 10.45 $\pm$ 0.73% ($p < .01$, Table 8). Differences in palmitoleate and stearate levels in the two groups disappeared. The extent of depression of EFA levels after 45 days was somewhat greater than after 21 days, although the characteristic EFA deficiency pattern was clearly established after only 21 days of EFA depletion. No C20:3ω9 was detectable in EFA sufficient animals, while the level rose in EFAD animals as the depletion continued.

T/T ratio

Triene:tetraene ratios for control animals were zero at both time points because no eicosatrienoate was detectable. After 21 days the T/T of the EFAD group was 0.26. After 45 days the T/T ratio had risen to 0.51.

Subjective observations

We observed no clinical signs of EFA deficiency. There was no difference in appearance of tail, skin, or hair of EFAD and control rats.

Experiment II

Body weights, gains, and food intake

Initial mean body weights among the groups at the commencement of the EFA depletion period ranged from 53 to 56g. At the end of the EFA depletion period animals were randomly assigned to experimental treatment groups. Analysis of variance confirmed no significant differences in body weight existed among groups. Mean body weights prior to application of the experimental dl-α-tocopheryl acetate doses ranged from 270 to 292g (Table 9). Final body weights were generally unresponsive to α-tocopheryl acetate dose (Figure 6). Final mean body weights of animals receiving 0, 1, 5, 10, or 20 mg dl-α-tocopheryl acetate/day were 456, 445, 479, 436, and 458g, respectively (Table 9).

Experimental weight gain ranged from 156 ± 9 to 187 ± 9g (Table 9). Food efficiency ratios declined with age from a mean of 0.467 during days 1 to 46 to a mean of 0.161 during days 80-91 (Table 9).

Serum fatty acid patterns

At the end of the EFA depletion period serum fatty acid patterns were similar among groups. Mean linoleate levels were 9.56% to 12.69% while T/T ratios were 0.47 to 0.82 (Table 10). The linoleate level and T/T ratio of the EFAD group of Experiment I fell within these ranges. Thus, the EFA depleted animals of Experiments I and II can be considered to be of the same population.

Dietary supplements of dl-α-tocopheryl acetate from 0 to 20 mg/day had no effect on the levels of individual fatty acids in total serum lipids (Table 11). Mean linoleate levels were 34.05% for the 1 mg

Table 9. Body weights, weight gains, and food efficiency ratios. Experiment II

Variable	Depletion period	Experimental period				
	EFAD	0-T	1-T	5-T	10-T	20-T
Initial body weight (g) (day 1)	54 ± 1[a]	56 ± 1	55 ± 1	56 ± 1	53 ± 1	54 ± 1
Body weight at end of EFA depletion (g) (45 days)	283 ± 3	281 ± 3	277 ± 3	292 ± 3	280 ± 3	270 ± 3
Body weight at end of experimental period (g) (91 days)	–	456 ± 10	445 ± 10	479 ± 10	436 ± 10	458 ± 10
Experimental period weight gain (g) (days 46-91)	–	174 ± 9	168 ± 9	187 ± 9	156 ± 9	187 ± 9
Depletion period FER (days 1-45)	0.462	0.462	0.479	0.471	0.466	0.462
Early experimental FER (days 46-61)	–	0.402	0.372	0.376	0.330	0.405
Middle experimental FER (days 62-79)	–	0.266	0.259	0.263	0.246	0.254

[a]Mean ± SEM.

Table 9. Continued

Variable	Depletion period	Experimental period				
	EFAD	0-T	1-T	5-T	10-T	20-%
Late experimental FER (days 80-91)	–	0.156	0.152	0.181	0.132	0.186

ANOVA indicated no significant differences due to supplemental level of dl-α-tocopheryl acetate.

Results of t-test: Experimental period weight gain, $p < .10$, group 10-T vs 20-T.

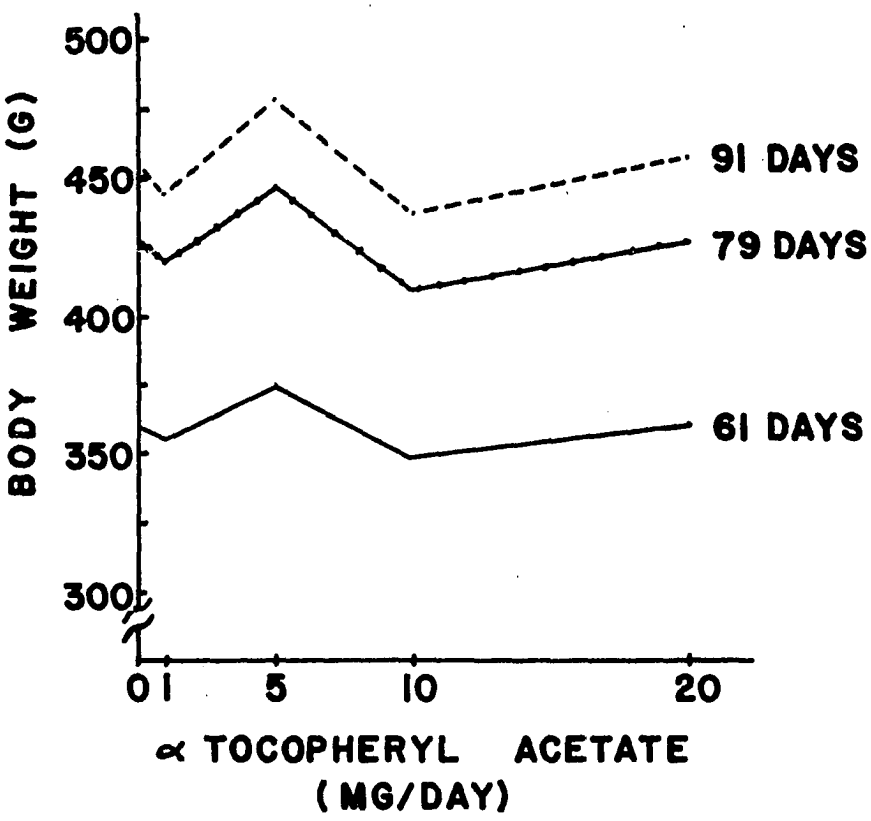

Figure 6. Dose-response curve of body weight at five α-tocopheryl acetate levels. Experiment II

Table 10. Serum fatty acid profiles at end of depletion period. Experiment II

Fatty acid	% of total fatty acids measured					
	EFAD	0-T	1-T	5-T	10-T	20-T
16:0	23.16 ± 0.89[a]	25.03 ± 1.03	26.70 ± 1.03	23.70 ± 1.03	24.07 ± 1.03	26.34 ± 1.03
16:1	3.38 ± 0.45	2.90 ± 0.47	2.96 ± 0.47	2.99 ± 0.47	2.36 ± 0.47	2.86 ± 0.47
18:0	22.53 ± 1.08	21.45 ± 0.97	23.20 ± 0.97	23.21 ± 0.97	24.69 ± 0.97	22.91 ± 0.97
18:1	21.56 ± 0.91	20.44 ± 1.00	21.31 ± 1.00	20.74 ± 1.00	18.81 ± 1.00	21.73 ± 1.00
18:2ω6	10.46 ± 0.81	10.83 ± 1.05	9.56 ± 1.05	11.59 ± 1.05	12.69 ± 1.05	10.39 ± 1.05
18:3ω3	Trace	Trace	Trace	Trace	Trace	Trace
20:3ω9	6.05 ± 0.67	7.31 ± 0.66	6.67 ± 0.66	6.70 ± 0.66	5.30 ± 0.66	6.05 ± 0.66
20:4ω6	12.84 ± 1.30	12.05 ± 1.28	9.59 ± 1.28	11.06 ± 1.28	12.09 ± 1.28	9.70 ± 1.28
T/T	0.50 ± 0.11	0.59 ± 0.11	0.82 ± 0.11	0.61 ± 0.11	0.47 ± 0.11	0.67 ± 0.11

ANOVA including all six groups showed no significant differences.

[a]Mean ± SEM.

Table 11. Serum total lipid fatty acid profiles at conclusion of Experiment II

Fatty acid	% of total fatty acids measured					
	Depletion period	Experimental period				
	EFAD	0-T	1-T	5-T	10-T	20-T
16:0	23.16 ± 0.89[a]	14.65 ± 0.98	15.08 ± 0.98	14.91 ± 0.98	13.74 ± 0.98	14.96 ± 0.98
16:1	3.38 ± 0.45	0.04 ± 0.01	0.06 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
18:0	22.53 ± 1.08	11.64 ± 0.76	12.41 ± 0.76	12.24 ± 0.76	11.77 ± 0.76	11.89 ± 0.76
18:1	21.56 ± 0.91	6.45 ± 0.93	6.41 ± 0.93	7.03 ± 0.93	6.11 ± 0.93	5.57 ± 0.93
18:2ω6	10.46 ± 0.81	36.13 ± 2.73	34.05 ± 2.73	32.55 ± 2.73	36.89 ± 2.73	33.95 ± 2.73
18:3ω3	Trace	0.05 ± 0.02	0.02 ± 0.02	0	0	0
20:3ω9	6.05 ± 0.67	0.17 ± 0.06	0.07 ± 0.03	0.13 ± 0.06	0.24 ± 0.10	0.15 ± 0.06
20:4ω6	12.84 ± 1.30	30.88 ± 2.29	31.92 ± 2.29	33.08 ± 2.29	31.21 ± 2.29	33.44 ± 2.29
T/T	0.50 ± 0.11	0.01	0	0	0.01	0

ANOVA showed no significant differences due to α-tocopheryl acetate supplementation.

[a]Mean ± SEM.

dl-α-tocopheryl acetate group and 33.95% for the 20 mg group. Mean arachidonate levels were 31.92% for the 1 mg group and 33.44% for the 20 mg group (Table 11).

Refeeding the EFA depleted animals with 20% SO lowered saturated and ω9 unsaturated fatty acids. Linoleate and arachidonate were increased threefold by SO refeeding (Table 11). Linoleate in the EFAD group was 10.46 ± 0.81% and in the 1-T group was 34.05 ± 2.73%. Arachidonate in the EFAD group was 12.84 ± 1.30% and in the 1-T group was 31.92 ± 2.29% (Table 11).

Pulmonary fatty acid profile

After EFA depletion C18:2ω6 was 2.40 ± 1.18% and C20:4ω6 was 10.48 ± 0.66% in lung (Table 12). Following refeeding with SO fatty acid composition of lung changed considerably, but values were not different due to dose level of α-tocopheryl acetate (Table 12). The 1-T and 20-T groups had 31.81 ± 1.98% and 30.93 ± 1.98% linoleate, respectively, and 13.59 ± 0.89% and 13.74 ± 0.89% arachidonate.

T/T ratios

At the end of the 45-day EFA depletion period serum T/T was greater than 0.4 in all groups, ranging from 0.82 ± 0.11 to 0.47 ± 0.11 (Table 10). After the 45-day refeeding period with 20% SO and five levels of dl-α-tocopheryl acetate all groups had normal T/T ratios in serum and pulmonary total lipids (Tables 11 and 12). The T/T ratios after feeding safflower oil and vitamin E ranged from 0.09 in lung to 0 in serum, similar to the 0 ratio in the control group of Experiment I (Table 13).

Table 12. Pulmonary total lipid fatty acid profile. Experiment II

Fatty acid	% of total fatty acids measured						
	Depletion period	Experimental period					ANOVA
	EFAD	0-T	1-T	5-T	10-T	20-T	FS T
16:0	42.90 ± 0.98[a]	30.93 ± 1.18	30.25 ± 1.18	31.93 ± 1.18	32.52 ± 1.18	30.92 ± 1.18	$p < .01$
16:1	1.33 ± 0.72	0.12 ± 0.52	0.15 ± 0.52	0.28 ± 0.52	0.21 ± 0.52	0.30 ± 0.52	
18:0	12.29 ± 0.46	10.85 ± 0.55	10.06 ± 0.55	10.29 ± 0.55	10.74 ± 0.55	10.35 ± 0.55	
18:1	29.17 ± 1.01	13.12 ± 0.70	13.60 ± 0.70	13.84 ± 0.70	13.96 ± 0.70	13.37 ± 0.70	$p < .01$
18:2ω6	2.40 ± 0.28	30.67 ± 1.98	31.81 ± 1.98	32.43 ± 1.98	28.96 ± 1.98	30.93 ± 1.98	$p < .01$
18:3ω3	Trace	Trace	Trace	Trace	Trace	Trace	
20:3ω9	1.41 ± 0.16	0.79 ± 0.23	0.55 ± 0.23	0.42 ± 0.23	0.34 ± 0.23	0.36 ± 0.23	$p < .01$
20:4ω6	10.48 ± 0.66	13.51 ± 0.89	13.59 ± 0.89	13.24 ± 0.89	13.27 ± 0.89	13.74 ± 0.89	
T/T	0.14 ± 0.02	0.09 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	

[a]Mean ± SEM.

Table 13. T/T ratios in serum and lung at end of depletion and refeeding periods. Experiment II

	EFAD	0-T	1-T	5-T	10-T	20-T
After 45-day depletion						
Serum	0.50 ± 0.11	0.59 ± 0.11	0.82 ± 0.11	0.61 ± 0.11	0.47 ± 0.11	0.67 ± 0.11
Lung	0.14 ± 0.02	–	–	–	–	–
After 45-day refeeding						
Serum	–	0.01	0	0	0.01	0.01
Lung	–	0.09 ± 0.03	0.04 ± 0.03	0.03 ± 0.03	0.03 ± 0.03	0.03 ± 0.03

T/T ratio of serum of control group in Experiment I was 0.

Hemolysis

In all experimental groups percent hemolysis was normal, ranging from 4.4 ± 1.2% to 7.6 ± 1.1% (Table 14). Even a presumptively deficient level of vitamin E (0 dietary supplementation) did not result in increased erythrocyte hemolysis (6.8 ± 1.1%). No differences were seen at any α-tocopheryl acetate dose.

Lung weight

Animals in the 10-T group had smaller lungs than animals in other groups (Table 14). However, when lung weight was based on body weight (g lung/100g B.W.), there were no longer significant differences by ANOVA. Groups 1-T and 10-T differed significantly on a t-test ($p < .01$, Table 14). Values for lung weight per 100g body weight ranged from 0.41 ± 0.02 for the 10-T group to 0.59 ± 0.04 for the 1-T group.

Pulmonary α-tocopherol

Dietary α-tocopherol level was reflected in tissue α-tocopherol concentration. Accumulation of α-tocopherol in lungs of animals fed increasing amounts of α-tocopheryl acetate was statistically significant (Table 14). Values in μg α-tocopherol/g wet lung tissue were 46.7 ± 4.7 and 63.2 ± 4.7 for groups 1-T and 20-T, respectively. A linear regression model fitting pulmonary α-tocopherol to dietary dl-α-tocopheryl acetate was significant at $p < .01$, although the low r^2 suggested a poor fit (Figure 7).

Table 14. Erythrocyte hemolysis, pulmonary weight and α-tocopherol content. Experiment II

	Depletion period	Experimental period	
	EFAD	0-T	1-T
RBC hemolysis (%)	4.4 ± 1.2[a]	6.8 ± 1.1	5.9 ± 1.1
Lung weight (g)	1.38 ± 0.08	2.11 ± 0.17	2.54 ± 0.17
g lung/100g body weight	0.49 ± 0.03	0.46 ± 0.04	0.59 ± 0.04
Pulmonary α-tocopherol (μg/g lung)	58.9 ± 3.8	49.2 ± 3.8	46.7 ± 3.8

Results of t-test:

Lung weight, 1-T vs 10-T, $p < .05$.
EFAD vs 1-T, $p < .05$.

Pulmonary α-tocopherol, 1-T vs 20-T, $p < .05$.

g lung/100g body weight, 1-T vs 10-T, $p < .01$.

[a]Mean ± SEM.

Experimental period			ANOVA	
5-T	10-T	20-T	FS	T
7.2 ± 1.1	7.6 ± 1.1	7.6 ± 1.1		
2.30 ± 0.17	1.78 ± 0.09	2.26 ± 0.23	$p < .05$	$p < .05$
0.48 ± 0.04	0.41 ± 0.02	0.49 ± 0.04		
59.0 ± 3.8	55.3 ± 3.8	63.2 ± 3.8		$p < .05$

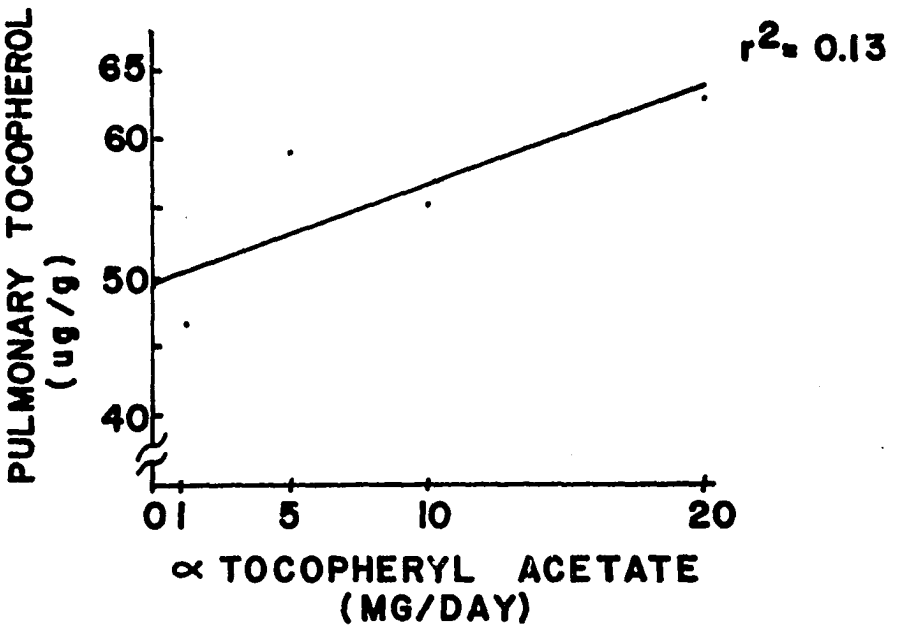

Figure 7. Linear regression of pulmonary α-tocopherol on dietary α-tocopheryl acetate. Experiment II

Experiment III

Body weights, gains, and food intake

By design mean body weights of all groups at commencement and conclusion of EFA depletion period were similar to one another (Table 15) and to groups from Experiments I and II.

During the experimental feeding of SO and graded levels of dl-α-tocopheryl acetate from 0 to 50 mg/day no differences in body weight appeared due to dose of vitamin E. The experimental time course was divided into early, middle, and late periods in order to monitor altering responses with progress of the experiment. At no time was there a statistically significant difference in body weight (Table 15). Weight gain during the late experimental period (days 83 to 91) tended to be reduced in the 50-T group, and total body weight showed a slight depression appearing only at the termination of the experiment (Figure 8). Final body weights ranged from 448 ± 10g to 466 ± 10g.

A food efficiency ratio was determined only once during the experiment, in the middle experimental period (days 76-82). Food efficiency ratios for the 0-T, 1-T, and 50-T groups were similar with values of 0.138, 0.116, and 0.102, respectively (Table 15).

Serum fatty acid pattern

At the end of the EFA depletion period the EFAD group was significantly different from the other groups in levels of palmitate and oleate (Table 16). The three groups to receive the experimental treatment, however, were uniform, with linoleate ranging from 5.38 ± 0.55% to 6.94 ± 0.55%, eicosatrienoate 7.77 ± 0.78% to 9.24 ± 0.78%, and

Table 15. Body weights, weight gains, and food efficiency. Experiment III

	Depletion period	Experimental period		
	EFAD	0-T	1-T	50-T
Initial body weight (g) (1 day)	51 ± 1[a]	52 ± 1	51 ± 1	52 ± 1
Body weight at end of EFA depletion (g) (45 days)	297 ± 5	303 ± 5	298 ± 5	297 ± 5
Body weight at end of experimental period (g) (91 days)	–	466 ± 10	458 ± 10	448 ± 10
Depletion period weight gain (g) (days 1-45)	246 ± 4	251 ± 4	246 ± 4	245 ± 4
Experimental period weight gain (g) (days 46-91)	–	163 ± 9	161 ± 9	151 ± 9
Early experimental weight gain (g) (days 46-76)	–	131 ± 6	135 ± 6	134 ± 6

[a]Mean ± SEM.

Table 15. Continued

	Depletion period	Experimental period		
	EFAD	0-T	1-T	50-T
Middle experimental weight gain (g) (days 77-82)	–	12 ± 3	11 ± 3	9 ± 3
Late experimental weight gain (g) (days 83-91)	–	19 ± 2	15 ± 2	8 ± 7
FER (days 77-82)	–	0.138 ± 0.036	0.116 ± 0.036	0.102 ±0.036

ANOVA indicated no differences among groups.

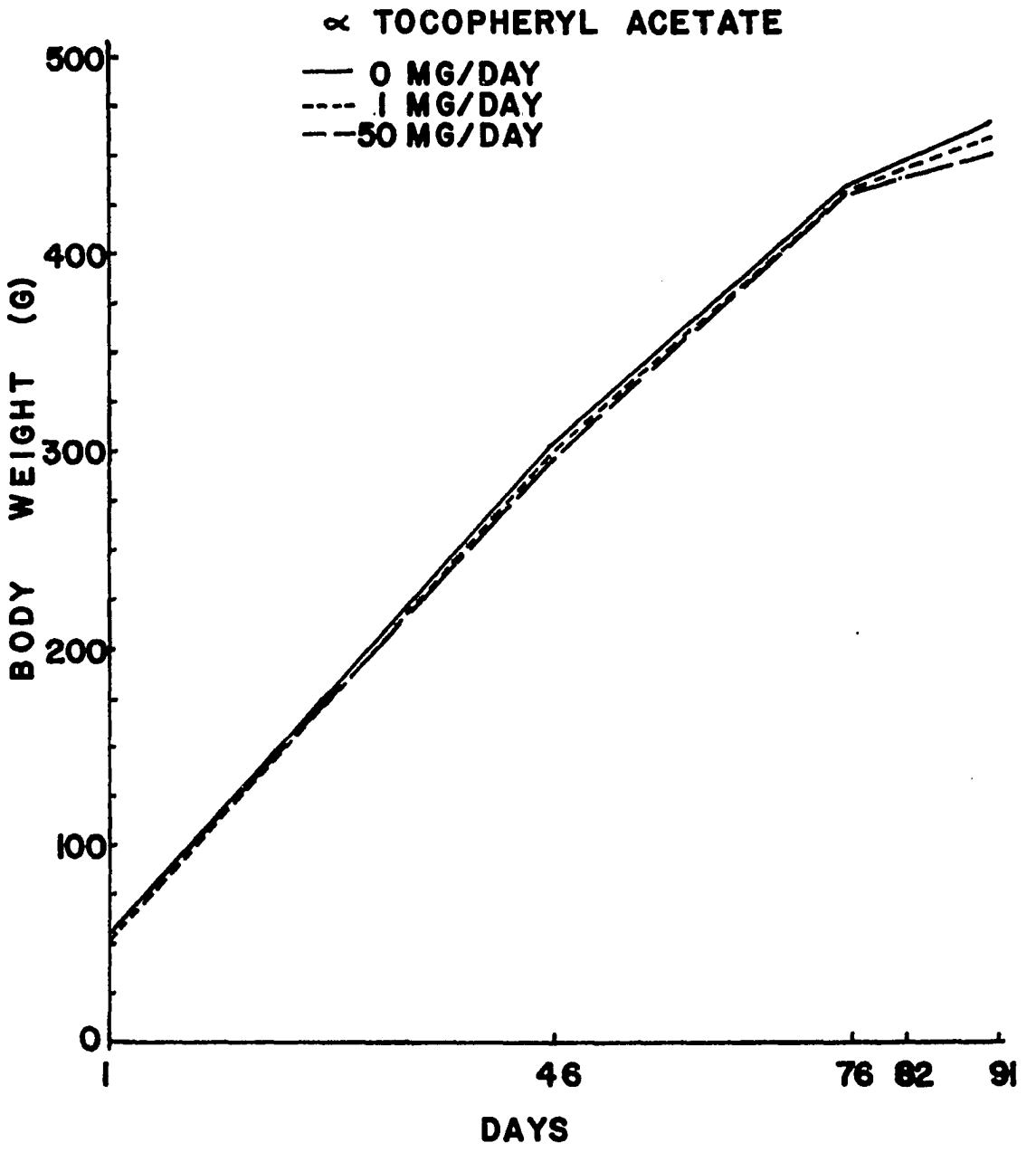

Figure 8. Body weights over time at three different doses of dl-α-tocopheryl acetate. Experiment III

Table 16. Serum total lipid fatty acid profile at end of depletion period (after 45 days). Experiment III

Fatty acid	% of total fatty acids measured				
	EFAD	0-T	1-T	50-T	ANOVA
16:0	29.77 ± 1.06[a]	26.54 ± 1.06	24.43 ± 1.06	25.36 ± 1.06	$p < .05$
16:1	4.01 ± 0.67	3.24 ± 0.67	2.22 ± 0.67	2.59 ± 0.67	
18:0	18.58 ± 0.91	17.28 ± 0.91	17.46 ± 0.91	16.53 ± 0.91	
18:1	24.04 ± 1.34	27.38 ± 1.34	29.24 ± 1.34	29.43 ± 1.34	$p < .05$
18:2ω6	7.04 ± 0.55	6.94 ± 0.55	5.38 ± 0.55	6.00 ± 0.55	
18:3ω3	Trace	Trace	Trace	Trace	
20:3ω9	6.57 ± 0.78	7.77 ± 0.78	9.24 ± 0.78	8.24 ± 0.78	
20:4ω6	9.98 ± 0.91	10.84 ± 0.91	12.05 ± 0.91	11.86 ± 0.91	
T/T	0.70 ± 0.09	0.75 ± 0.08	0.83 ± 0.08	0.71 ± 0.08	

ANOVA included all four groups.

[a]Mean ± SEM.

arachidonate 10.84 ± 0.91% to 12.05 ± 0.91%. The T/T ratios showed all four groups uniformly EFA deficient with T/T = 0.70, 0.75, 0.83, and 0.71 (Table 16).

Level of daily dietary supplementation with dl-α-tocopheryl acetate was of no significant effect on serum total lipid fatty acid profile on the 91st experimental day (Table 17). However, group 50-T, which received 50 mg dl-α-tocopheryl acetate/day, tended to have a reduced level of serum arachidonate. Group 1-T had 25.02 ± 1.76% C20:4ω6 while group 50-T had 18.75 ± 1.76%. This difference approached

Table 17. Serum total lipid fatty acid profile at end of Experiment III

Fatty acid	% of total fatty acids measured			
	Depletion period	Experimental period		
		0-T	1-T	50-T
16:0	29.77 ± 1.06[a]	20.23 ± 1.69	22.47 ± 1.69	20.74 ± 1.69
16:1	4.01 ± 0.67	0.56 ± 0.28	0.72 ± 0.28	0.84 ± 0.28
18:0	18.58 ± 0.91	13.62 ± 1.02	14.78 ± 1.02	14.51 ± 1.02
18:1	24.04 ± 1.34	7.26 ± 0.64	6.78 ± 0.64	7.72 ± 0.64
18:2ω6	7.04 ± 0.55	30.20 ± 2.32	22.86 ± 2.61	30.96 ± 4.19
20:3ω9	6.57 ± 0.78	4.44 ± 0.97	7.36 ± 0.97	6.49 ± 0.97
20:4ω6	9.98 ± 0.91	23.69 ± 1.76	25.02 ± 1.76	18.75 ± 1.76
T/T	0.70 ± 0.09	0.20 ± 0.04	0.28 ± 0.04	0.31 ± 0.04

ANOVA indicated no significant differences due to α-tocopheryl acetate supplementation.

[a]Mean ± SEM.

significance at $p < .05$. The t-test indicated no difference in C18:2ω6 for groups 1-T and 50-T. The mean T/T ratio of the EFAD group was 0.70. The mean T/T ratio of the SO refed groups was 0.26 ± 0.04 (Table 17), higher than the 0.03 in the SO refed groups of Experiment II (Table 12).

Pulmonary fatty acid profile

Levels of daily dietary vitamin E supplementation had no significant effect on pulmonary total lipid fatty acid profile on the 91st experimental day (Table 18). Linoleate in group 1-T was 31.93 ± 1.67% and in group 50-T 33.54 ± 1.67%. Arachidonate in group 1-T was

Table 18. Pulmonary total lipid fatty acid profile. Experiment III

Fatty acid	% of total fatty acids measured				
	Depletion period	Experimental period			ANOVA
		0-T	1-T	50-T	FS T
16:0	33.77 ± 0.77[a]	27.88 ± 0.71	28.12 ± 0.71	26.78 ± 0.71	$p < .01$
16:1	6.37 ± 0.20	1.18 ± 0.19	0.92 ± 0.19	0.90 ± 0.19	$p < .01$
18:0	14.71 ± 0.42	11.89 ± 0.39	11.63 ± 0.39	11.36 ± 0.39	$p < .01$
18:1	23.26 ± 0.54	12.54 ± 0.44	12.91 ± 0.44	13.14 ± 0.44	$p < .01$
18:2ω6	3.72 ± 1.58	32.26 ± 1.67	31.93 ± 1.67	33.54 ± 1.67	$p < .01$
18:3ω3	Trace	Trace	Trace	Trace	
20:3ω9	3.06 ± 0.18	0.75 ± 0.06	0.79 ± 0.06	0.74 ± 0.06	$p < .01$
20:4ω6	14.94 ± 1.00	13.39 ± 1.08	13.66 ± 1.08	13.48 ± 1.08	
T/T	0.20	0.06	0.06	0.05	

[a]Mean ± SEM.

13.66 ± 1.08% and in group 50-T 13.48 ± 1.08%. The T/T ratios of the EFAD, 0-T, 1-T, and 50-T groups were 0.20, 0.06, 0.06, and 0.05, respectively.

Another perspective on the distribution of fatty acids within the total lipids of a tissue is gained by calculation of ratios of selected fatty acids. While EFA deficiency was of significant effect in raising C18:1/C18:0, C20:4ω6/C18:2ω6, C20:3ω9/C20:4ω6 + C18:2ω6 (T/T + D) in lung and serum, supplementation with α-tocopheryl acetate had no effect on the pulmonary ratios (Table 19). Dietary α-tocopheryl acetate

Table 19. Selected pulmonary and serum fatty acid ratios. Experiment III

	Depletion period	Experimental period				
	EFAD	0-T	1-T	50-T	ANOVA FS	ANOVA T
Lung						
C18:1/C18:0	1.63 ± 0.09[a]	1.11 ± 0.08	1.15 ± 0.08	1.18 ± 0.08	$p < .01$	
C20:4ω6/C18:2ω6	5.36 ± 0.35	0.46 ± 0.06	0.50 ± 0.06	0.42 ± 0.06	$p < .01$	
C20:3ω9/C20:4ω6 + C18:2ω6 (T/T + D)	0.17 ± 0.01	0.02 ± 0.0	0.02 ± 0	0.02 ± 0	$p < .01$	
Serum						
C18:1/C18:0	1.37 ± 0.10	0.56 ± 0.08	0.49 ± 0.08	0.64 ± 0.08	$p < .01$	
C20:4ω6/C18:2ω6	1.53 ± 0.14	0.88 ± 0.10	1.21 ± 0.10	0.73 ± 0.10	$p < .01$	$p < .05$
C20:3ω9/C20:4ω6 + C18:2ω6 (T/T + D)	0.40 ± 0.03	0.08 ± 0.02	0.15 ± 0.03	0.14 ± 0.02	$p < .01$	

[a]Mean ± SEM.

significantly decreased the serum C20:4ω6/C18:2ω6 ratio ($p < .05$). The serum ω6 ratios for groups 1-T and 50-T were 1.21 ± 0.10 and 0.73 ± 0.10 (Table 19).

Lung weight

As in Experiment II, when lung weight was expressed relative to body weight, vitamin E treatment showed no effect (ANOVA). The proportion of lung to 100g body weight was similar in both experiments (Tables 14 and 20). The proportional lung values for the 1-T and 50-T groups of Experiment III were 0.52 ± 0.04 and 0.47 ± 0.04 (Table 20).

Pulmonary α-tocopherol

Pulmonary α-tocopherol tended to increase with additional α-tocopheryl acetate in the diet, although the increase was not statistically significant. Concentrations of α-tocopherol in the lung for groups receiving 0, 1, or 50 mg dl-α-tocopheryl acetate/day were 54.4 ± 3.7, 57.7 ± 3.7, and 63.3 ± 3.7 μg/g lung, respectively (Table 20). Linear regression analysis revealed that although the data could be fitted to a straight line with positive slope, relating dietary and tissue α-tocopherol, the slope of the line was not statistically different from zero (Figure 9). Thus, although there was an apparent trend toward pulmonary tocopherol accumulation, it was not significant.

Serum prostaglandins

The EFAD group had significantly reduced synthesis of PGE_1 but not of PGE_2 or $PGF_{2\alpha}$ as compared to the SO refed groups. The average

Table 20. Pulmonary weight and α-tocopherol content. Experiment III

	Depletion period	Experimental period			ANOVA	
	EFAD	0-T	1-T	50-T	FS	T
Lung weight (g)	1.42 ± 0.18[a]	2.04 ± 0.19	2.38 ± 0.19	2.07 ± 0.19	$p < .01$	
g lung/100g body weight	0.48 ± 0.04	0.42 ± 0.04	0.52 ± 0.04	0.47 ± 0.04		
α-tocopherol level (μg/g lung)	67.8 ± 4.9	54.4 ± 3.7	57.7 ± 3.7	63.3 ± 3.7		

[a]Mean ± SEM.

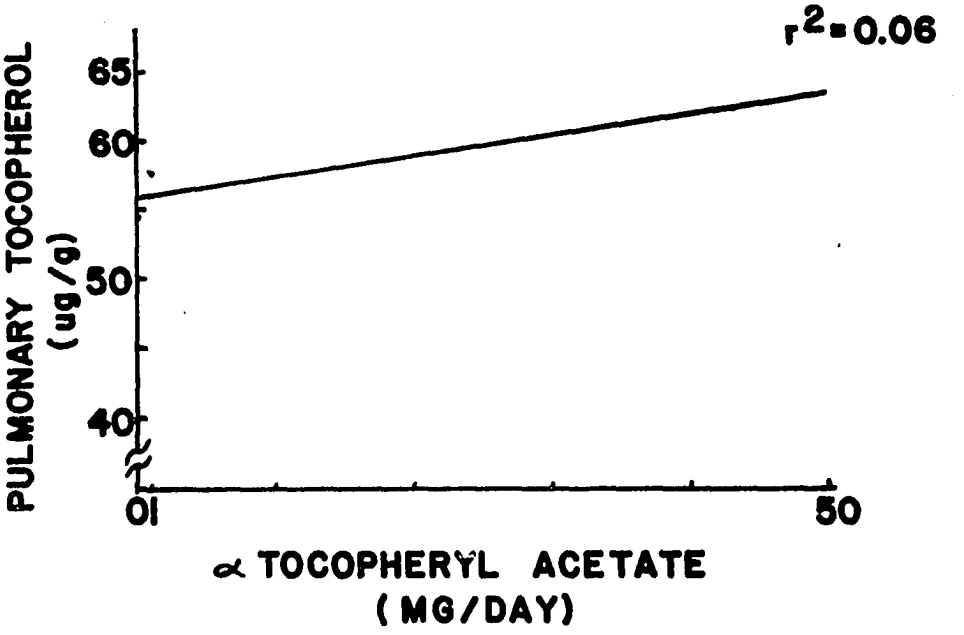

Figure 9. Linear regression model for pulmonary α-tocopherol upon dietary α-tocopheryl acetate. Experiment III

PGE_1 value of the three experimental groups was twice that of the EFAD group. Mean PGE_1 for group EFAD and group 0-T were 0.46 and 1.19 ng/ml (Table 21). Of the three PGs measured, $PGF_{2\alpha}$ was present in the highest concentrations and PGE_1 in the lowest in all groups. In group EFAD $PGF_{2\alpha}$ was 9.66 ± 2.61 ng/ml, PGE_2 was 2.72 ± 0.99 ng/ml, and PGE_1 was 0.46 ± 0.15 ng/ml. Comparison of groups 0-T, 1-T, and 50-T revealed no differences in synthesis of PGE_1, PGE_2, or $PGF_{2\alpha}$ in incubated whole blood (Table 21).

Pulmonary prostaglandin synthesis

Essential FA deficiency did not reduce PG synthesis in pulmonary homogenate. On the contrary, PGI_2 synthesis was significantly higher in lungs of the EFAD group than in groups 0-T, 1-T, and 50-T. Supplementation with dl-α-tocopheryl acetate had no effect on PG synthesis in lung homogenate (Table 22). In groups 1-T and 50-T PGE_1 was 0.08 ± 0.01 and 0.07 ± 0.01 ng/mg, respectively. The levels of PGE_2 measured in groups 1-T and 50-T were 0.28 ± 0.02 and 0.31 ± 0.02 ng/mg. Prostaglandin $F_{2\alpha}$ in groups 1-T and 50-T was 7.61 ± 0.94 and 7.58 ± 0.94 ng/mg lung. Prostacyclin in group 1-T was 1.84 ± 0.16 and in group 50-T 1.86 ± 0.16 ng/mg. The $PGE_1/PGE_2 + PGF_{2\alpha}$ ratio in lung was unaffected by dietary treatment (Table 22). Vitamin E supplementation did not alter the balance among PGs of the one and two series.

Table 21. Prostaglandin synthesis in 10-minute incubated arterial whole blood. Experiment III

	Depletion period	Experimental period			ANOVA	
	EFAD	0-T	1-T	50-T	FS	T
PGE_1 (ng/ml serum)	0.46 ± 0.15[a]	1.19 ± 0.15	0.69 ± 0.15	0.92 ± 0.15	$p < .05$	
PGE_2 (ng/ml serum)	2.72 ± 0.99	3.73 ± 0.99	5.50 ± 0.99	4.41 ± 0.99		
$PGF_{2\alpha}$ (ng/ml serum)	9.66 ± 2.61	14.75 ± 2.61	14.23 ± 2.61	14.02 ± 2.61		
$\frac{PGE_1}{PGE_2 + PGF_{2\alpha}}$	0.05 ± 0.01	0.10 ± 0.03	0.07 ± 0.01	0.06 ± 0.01		

[a]Mean ± SEM.

Table 22. Prostaglandin synthesis in 10-minute incubated lung homogenate. Experiment III

	Depletion period	Experimental period			ANOVA	
	EFAD	0-T	1-T	50-T	FS	T
PGE_1 (ng/mg lung)	0.08 ± 0.01[a]	0.08 ± 0.01	0.08 ± 0.01	0.07 ± 0.01		
PGE_2 (ng/mg lung)	0.25 ± 0.02	0.28 ± 0.02	0.28 ± 0.02	0.31 ± 0.02		
$PGF_{2\alpha}$ (ng/mg lung)	8.23 ± 0.94	7.06 ± 0.94	7.61 ± 0.94	7.58 ± 0.94		
PGI_2 (ng/mg lung)	2.36 ± 0.16	1.65 ± 0.16	1.84 ± 0.16	1.86 ± 0.16	$p < .05$	
$\frac{PGE_1}{PGE_2 + PGF_{2\alpha}}$	0.01	0.02	0.01	0.01		

[a]Mean ± SEM.

DISCUSSION

Hypothesis

Four disparate observations from past research form the basis for the present work.

1. High PUFA diets may have deleterious effects.
2. Level of dietary PUFA may influence PG synthesis.
3. Vitamin E may inhibit PG synthesis in vitro.
4. Vitamin E requirement is influenced by PUFA intake.

Synthesis of these four ideas suggests that the deleterious effects of high PUFA diets may be mediated by altered PG synthesis coupled with a secondary vitamin E deficiency. An increase in dietary vitamin E might ameliorate the ill effects of high PUFA diets by moderating PG synthesis.

The purpose of the present investigation was to test the interrelationship of dietary vitamin E, dietary linoleic acid, and prostaglandin synthesis. Alpha-tocopheryl acetate, one of the most stable forms of vitamin E, was used in vitamin supplements. A high dietary intake of safflower oil gave a rich source of linoleate.

Model for Dietary Linoleate Research

In order to observe the effects of dietary α-tocopherol on ingested C18:2ω6 rather than on endogenous C18:2ω6, I attempted to reduce the contribution of tissue to metabolic PUFA pools and maximize the contribution of diet. Animals were maintained on an EFA deficient diet for 45 days to deplete tissue stores of 18:2ω6. Reid *et al.* (1968) estimated that a young rat, fed *ad libitum* for 14 days, would have

10% of body weight as fat. This is admittedly a general figure, as composition of the diet, length of feeding period, and age of the rat would affect the value. Carlson and Arnrich (1978) estimated a 350g adult rat would have an average of 30% of body weight as lipid. According to their estimates, rats in this study weighing ca. 300g at the end of the depletion period would have a body store of ca. 30-90g lipid. Although this fat is in various metabolic pools with varying turnover rates, one may use again the general rate of Reid et al. of 12% turnover/day. Thus, 3-10g fat is turning over daily. At the end of EFA depletion we found 10-12% linoleate in serum and 2-4% in lung. Coniglio et al. (1976) reported hepatic 18:2ω6 in EFA deficiency was 6.6% of total fatty acids. Using an average figure of 7% of body fat as 18:2ω6 in EFA deficiency, the tissue contribution of metabolizable 18:2ω6 was no more than 0.25-0.77g/day at the end of the depletion period. On a 20% SO diet our rats were ingesting ca. 14g diet/day or 2.8g SO, which by our analysis contained 18:2ω6 at 81% of total fatty acids. Thus, the dietary contribution of linoleate was 2.27g/day. At the commencement of the experimental phase, when the dietary fat was changed from hydrogenated coconut oil (HCO) to safflower oil (SO), the dietary contribution of 18:2ω6 to labile pools was four to ten times the tissue contribution. Towards the end of the SO feeding regimen, body stores of 18:2ω6 were high. Linoleate was 35% of serum total lipids and 30% of pulmonary total lipids. By calculations, therefore, the tissue contribution of C18:2ω6 was estimated to be 1.1-3.8g/day. Dietary contribution of C18:2ω6 to labile pools still rivalled tissue contribution.

Use of a 26% casein, 20% HCO diet produced biochemical but not clinical symptoms of EFA deficiency within 45 days. The EFA deficient rats appeared normal in all respects except in T/T ratio of serum total lipids. There was neither growth depression nor dermal abnormality in the EFA deficient rats. After only 22 days of an EFA deficient diet the T/T ratio of serum total lipids was already 0.26 as compared to 0 for control animals. After 45 days the T/T ratio of serum total lipid was 0.51, just within the biochemical definition of EFA deficiency (≥ 0.4). At the end of the 45-day EFA depletion period in Experiments II and III, the serum total lipid T/T ratios averaged 0.58 and 0.71, respectively. Three separate trials, depleting over 110 animals, demonstrated the efficacy and reproducibility of the EFA deficient diet to produce the desired population.

Thus, it appears that a model for study of effects on dietary C18:2ω6 has been successfully established. This model is composed of young rats who are indistinguishable from healthy control rats in all gross respects measured except in tissue stores of PUFA. The rats show no growth retardation, appetite depression, or dermal lesions that could complicate subsequent experimental treatments and measures.

Effect of α-Tocopheryl Acetate on Body Weight, Growth, Food Efficiency

Feeding a 20% by weight SO diet to weanling rats for 28 days has previously been associated with reduced growth rate and reduced energy efficiency (Higgins 1979). In Experiment II we found that levels of

dietary α-tocopheryl acetate from 0 to 20 mg/day had no significant effect on body weight, weight gain, or food efficiency after 45 days of feeding. These rats, however, were older than those of Higgins when the dietary regimen with 20% SO was started. At an age of 111 days the rats weighed on the average 450g in the second experiment and 457g in the third experiment. Replication between experiments indicated consistent, reliable results. When graded levels of α-tocopheryl acetate (0, 1, 5, 10, and 20 mg/day) were administered, no clear trend of effect on body weight appeared. Final weights were randomly scattered about the mean with no relationship to treatment. When the α-tocopheryl acetate supplementation was increased to 50 mg/day in the third experiment, the final body weights of the high-vitamin E group tended to be depressed compared to the low-vitamin E group (Table 14). Although the effect was not statistically significant, it was beginning to appear only in the final days of the experiment. This late-appearing trend to growth depression suggests (1) the level of vitamin E in question — 50 mg/day — may have approached a toxic dose, and (2) the experiment ended too soon. Corrick (1969) found growth depression in rats fed over 75 IU α-tocopherol/day for ten weeks. Conditions in the present study were moderate by comparison and gave less dramatic results. The high-vitamin E supplement of 50 mg/day was approximately 3570 mg/kg diet. This is nearly 100 times the estimated requirement of the laboratory rat or mouse (National Research Council 1978). Any nutrient, given at such levels, could be considered potentially dangerous. It is possible the experiment ended before the effect was distinguishable. Future research might explore the long-term

consequences of high-vitamin E supplementation.

Body weight may mask subtle effects of diets and may be criticized, too, as a poor indicator of nutritive status (Morley 1976). A homogeneous population in regard to initial weight and depletion weight was used for further experimental applications. In the second experiment α-tocopheryl acetate supplementation had no statistically significant effect on weight gain over the 45-day experimental period. Table 9 shows a scatter of gains and no clear trend with increasing dietary α-tocopherol. This suggests the reported weight gains are truly random, and α-tocopheryl acetate supplementation of 0 to 20 mg/day did not modify weight gain. In the third experiment weight gain, although not statistically significant, appeared mildly depressed in the 50 mg/day vitamin E group (Table 15). The division of overall weight gain into early, middle, and late periods suggests that the reduced growth rate, if genuine, came only late in the experiment. Again, one wonders what continuation of the feeding period might have revealed.

The food efficiency ratio (FER) in Experiment III calculated during the middle experimental period (days 76-82), showed no statistically significant effect of dietary α-tocopheryl acetate, although as with body weight and weight gain, there was an apparent decline in FER with high vitamin E. As there was no significant depression in weight gain during the middle experimental period, the apparently reduced FER would be due to an increased food intake and decreased utilization. However, as no statistical evidence of experimental effect was evident, one must conclude that dietary vitamin E did not influence food efficiency.

Effect of Dietary α-Tocopheryl Acetate on Hemolysis

The percent of erythrocyte hemolysis is commonly used to assess vitamin E nutriture. Values of $< 10\%$ are considered to indicate adequacy of dietary vitamin E. The data indicate all six treatment groups had adequate α-tocopherol, and there was no increase in hemolysis with decreasing level of tocopherol supplementation (Table 14). The data indicate hemolysis to be an insensitive indicator of vitamin E intake. Severe deficiency would be signalled by this indicator, but subtle differences in α-tocopheryl acetate intake such as were employed in Experiment II are not reflected in hemolysis results. On the one hand, increasing levels of α-tocopheryl acetate above a moderate (1.0 mg/day) intake were not accompanied by changes in percent hemolysis. This suggests around 6% hemolysis is a background level, resistant to reduction or improvement. Thus, hemolysis is useless as an indicator of vitamin E intakes above adequate. We agree with Mackenzie (1954) that above a critical level of serum tocopherol, there is complete protection against the hemolytic response.

On the other hand, even the group receiving a zero vitamin E supplementation to the SO diet showed no perceptible increase in percent hemolysis. Although we expected this group to be vitamin E deficient, there is evidence they were not. In the semi-purified diet provided to the animals, only the SO could have been a source of vitamin E. The α-tocopherol content of SO is generally 0.34 mg/g oil (McLaughlin and Weihrauch 1979), although determination of the α-tocopherol content of our particular batch of SO revealed only half

as much α-tocopherol (0.112 mg/g oil). Discussions with the quality control manager of the SO manufacturer revealed the company used TBHQ as added antioxidant; tocopherol contents were believed low due to processing. Rats in these experiments, therefore, were receiving 0.31 mg α-tocopherol/day from SO alone or 22 mg/kg diet. Although the diet itself was not altogether void of α-tocopherol, the level present was presumptively deficient. Failing to observe symptoms of vitamin E deficiency, one must conclude that the minimal amount of α-tocopherol in the SO used was adequate to delay appearance of deficiency symptoms.

The purpose in the present investigation was not to produce a vitamin E deficiency, in any case. The O-T group was included as one part of a wide spectrum of levels of α-tocopheryl acetate supplementation. Although there was no intention to produce vitamin E deficiency, the results suggest two measures that would be useful in such a purpose: (1) use of a more completely stripped SO, treated with non-biological antioxidants, and (2) continuation of the O-T regimen exceeding 45 days.

The method of hemolysis analysis herein employed is based on spontaneous hemolysis in saline-phosphate buffer rather than the older dialuric acid method. The former method is more sensitive than the latter (Draper and Csallany 1969). Aftergood and Alfin-Slater (1978), using the Draper and Csallany method, reported RBC hemolysis of rats fed 1.0 mg/day or 5.0 mg/day α-tocopherol for 12 weeks was 4.5 $\pm$ 2.3% and 3.8 $\pm$ 1.4%, respectively. Comparable values from the present study are 5.9 $\pm$ 1.1% and 7.2 $\pm$ 1.1%, indicating agreement with Aftergood

and Alfin-Slater that increasing α-tocopherol was of no significant benefit in lowering erythrocyte hemolysis.

Although statistically not significant, the lowest RBC hemolysis value occurred in the EFAD group. To our knowledge hemolysis per se has not previously been measured in EFA deficiency. A group of investigators reported that osmotic resistance of erythrocyte membranes, measured by dropping whole blood into NaCl solutions of varying concentrations, was no different in EFA deficiency or adequacy (Bohles et al. 1979). Another study showed that rabbit RBCs were resistant to peroxidative hemolysis until arachidonate was added to the medium (Brin et al. 1974). The rabbits were not EFA deficient, although they were vitamin E deficient. One cannot conclude with assurance that in a vitamin E sufficient state, reduced PUFA would make the RBC membranes more stable, although the results indicate that is possible.

A reduced hemolytic response may be explained in at least two ways. (1) The EFAD group was 6 weeks younger than the other treatment groups. Thus, any difference observed between EFAD and the other groups may be an age rather than diet difference. Caution should be employed in making any direct comparisons. The age-related studies reported earlier show greater hemolysis in the young, but that is thought due to reduced α-tocopherol stores in the young (Gordon and Nitowsky 1956). Whether age, independent of α-tocopherol content of the body, affects hemolysis is unclear. (2) Hemolysis is a result of fragile red blood cell membranes, and membrane integrity depends in part upon structural lipids. Current theory holds that membranes, rich in PUFA, are structurally sound as long as the PUFA remains unoxidized.

If peroxidation occurs, the membrane is disrupted and hemolysis potential is increased. Alpha-tocopherol, as an antioxidant, can prevent the loss of membrane integrity. In EFAD it is possible (1) that membranes are depleted in PUFA, (2) that the ratio of E:PUFA is increased, (3) that the remaining scarce PUFA are more vigilantly protected from peroxidative damage.

Effect of Diet on Lung Weight

Size of the lung was affected by α-tocopherol supplementation. In Experiment II animals in the experimental group receiving 10 mg dl-α-tocopheryl acetate/day had significantly smaller lungs than animals in all other groups (Table 14). This is an intriguing and puzzling observation. Experimental group 10-T was a maverick on several variables measured. This group had the highest hemolysis value, lowest food efficiency, and lowest weight gain of all experimental groups refed with SO and graded levels of α-tocopheryl acetate. Group 10-T also had lower pulmonary C18:2ω6 and higher C16:0 than the other groups at the conclusion of the experiment. Initial body weight and weight at end of EFA depletion were not different from the other groups (Tables 9 and 14). Serum fatty acid profile of group 10-T was not different from the other groups at the end of depletion (Table 10). Thus, as far as can be known, no selection bias accounts for the data obtained for group 10-T. The differences were not large enough to be statistically significant by ANOVA or by t-tests but that this group deviated consistently from all other groups on so many variables

suggests something unique about the group. That the lungs were significantly smaller in group 10-T than in the other treatment groups may partially explain the poor showing on indices of health. A limited pulmonary capacity may restrict growth and general well-being. When lung size was considered relative to body weight (Table 14), groups 10-T and 1-T differed significantly.

If the treatment group receiving 20 mg α-tocopheryl acetate/day had responded in a similar manner to the group receiving 10 mg/day, then one may have concluded the effects were due to excessive α-tocopherol ingestion. That is not the case. A basic assumption in nutrition science is that every nutrient has a continuous effect upon the organism. As graphically portrayed in Figure 10, both deficiency and excess of a nutrient are accompanied by deleterious effects, while a middle, moderate range is associated with optimum functioning. If, for example, the nutrient in question were EFA and the response were growth, we would observe at suboptimal intakes a depressed growth, at optimal intakes maximum growth, and at excessive intakes depressed growth. Thus, in the case of α-tocopherol we would also expect to observe a continuous effect of increasing dose, a uniformly progressive decline or increase in lung weight, or weight gain, for example. What we report, however, is a break in the continuous curve. While it is conceivable that increasing α-tocopheryl acetate intake from 1 to 5 to 10 mg/day could lead to deleterious effects at the highest dose, what is inconceivable, given our current assumptions, is that a further increase to 20 mg/day should reverse the trend.[1] The continuous effect model is a simplified statement of nutrient effect ignoring the

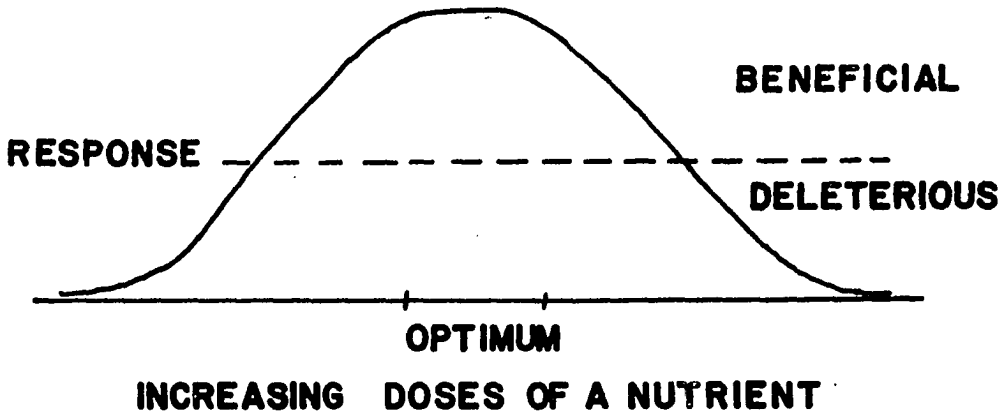

Figure 10. Generalized continuous response curve of a nutrient

reality that nutrients act in concert. The observations of group 10-T may be strictly random but may on the other hand result from an undefined nutrient interaction.

Pulmonary α-Tocopherol

Results show that increasing levels of α-tocopheryl acetate in the diet were associated with slightly increased concentrations of α-tocopherol in lung tissue. The increase in daily supplementation from 1 mg to 20 mg was accompanied by a rise in lung α-tocopherol from 46.7 μg/g to 63.2 μg/g lung. Although the increased pulmonary concentration was statistically significant, it was only a modest response to large dietary increases. Linear regression showed that a straight line was a poor fit to the data, primarily because of wide variation within groups. In the third experiment dietary α-tocopheryl acetate was raised to 50 mg/day. Pulmonary content of α-tocopherol under this treatment was 63.3 μg/g lung, no higher than when intake was 20 mg/day in Experiment II. Furthermore, the third experiment failed to show any statistical difference among treatment groups in pulmonary α-tocopherol content.

The effect of dietary α-tocopheryl acetate on pulmonary α-tocopherol accumulation is minor at best and straitly restricted. Although increased intake of α-tocopherol results in apparent accumulation in plasma and liver, the lung is less responsive (Aftergood and Alfin-Slater 1978). This finding is consistent with data related to lung composition. The total fat content of pulmonary tissue is

relatively small. But more important, 50% of pulmonary lipid is in the phospholipid fraction, and this fraction is of a relatively fixed composition, somewhat resistant to dietary changes (Higgins 1979). A small nonphospholipid pool in lung suggests a limited ability to accumulate and store fat-soluble vitamins.

Witting (1975) reported α-tocopherol was present in tissues largely in the subcellular membranes; therefore, incorporation and storage of α-tocopherol in tissues would be severely limited. Bieri et al. (1978) fed rats graded levels of α-tocopheryl acetate from 30 to 50 mg/kg diet for 8 to 10 weeks and found no change in the α-tocopherol content of lung.

Comparisons of pulmonary tocopherol data from the present investigation with earlier published results show close agreement with Aftergood and Alfin-Slater (1978) under similar conditions. That Evarts and Bieri's (1974) values of pulmonary α-tocopherol are much lower than Aftergood and Alfin-Slater's and the results herein may be due to two factors. (1) Evarts and Bieri did not supplement the corn oil diet with vitamin E as did the others. (2) Evarts and Bieri reported a value based on only two rats. Our results with over 100 rats indicated wide variation in the rat population and high error in a small sample.

It is evident from the SEM that a relatively high error persists in α-tocopherol measures (Tables 14 and 20). Such error is due to at least two factors: (1) a genuinely wide biological variability in the population and (2) error in the method. That the latter is partly to blame became clear as refinement of the method occurred during use. Only in the hands of a skilled analyst can the potential for variation

Table 23. Comparisons of literature values for pulmonary α-tocopherol in rats

	Evarts and Bieri (1974)	Aftergood and Alfin-Slater (1978)	Work herein
Pulmonary α-tocopherol (μg/g lung)	21.6	56.0 ± 5.6[a]	59.0 ± 3.8[a]
Diet	20% corn oil	15% corn oil 5 mg α-tocopherol/day	20% SO 5 mg tocopherol/day
Length of feeding	8-12 weeks	6 weeks	6 weeks

[a]Mean ± SEM.

be reduced. Alpha-tocopherol, unstable, readily oxidized by air, light, heat, was sensitive to even slight differences in sample handling and preparation, as indicated by duplicate disagreement.

In general, levels of α-tocopherol in lung reported here are similar to those reported by others, and little pulmonary retention of excessive α-tocopherol intakes occurred.

When EFAD animals are compared to the other treatment groups in Experiments II and III, the EFAD animals had a relatively high pulmonary α-tocopherol content despite an α-tocopheryl acetate intake of only 1.0 mg/day and despite an age difference of six weeks compared to other treatment groups. As has previously been mentioned, α-tocopherol levels are generally low in very young animals. At the ages in these experiments, 86 days old (EFAD group) vs 111 days old (SO and α-tocopheryl acetate treated groups), the age effect on tissue α-tocopherol content is not known. In the absence of a proper control group for comparison with the EFAD group one cannot make clear evaluation of the EFAD results. Nevertheless, it appears that EFAD animals have increased pulmonary α-tocopherol (Tables 14 and 20). Although plasma α-tocopherol was not measured, the high pulmonary α-tocopherol values are consistent with the trend towards reduced hemolysis seen in EFAD animals. In a state of EFA deficiency (1) the organism might compensate for EFA scarcity by accumulating protective molecules, or (2) reduced substrate for lipid oxidation might diminish use of the vitamin and enhance accumulation.

Serum Fatty Acid Pattern

Rats entered the experimental phase having been uniformly depleted of EFA. Mean serum T/T ratios in Experiments II and III ranged from 0.47 to 0.83; all within the biochemical definition of EFA deficiency. Serum levels of C18:2ω6 and C20:4ω6 were reduced to half of control levels (Table 8). At this juncture all rats to be continued in the experiment were placed on a 20% by weight SO diet, with 31% of calories supplied by C18:2ω6. The excess C18:2ω6 intake was accompanied by 0, 1, 5, 10, or 20 mg/day dl-α-tocopheryl acetate in Experiment II or by 0, 1, or 50 mg/day dl-α-tocopheryl acetate in Experiment III. The levels of the α-tocopheryl acetate in supplements in Experiment II had no statistically significant effect on any of the fatty acids measured. Apparently vitamin E did not influence the conversion of dietary linoleate to arachidonate. Data from Experiment III partially confirm these results. Statistical analysis shows dietary α-tocopherol level did not affect serum fatty acid profile (Table 17). Closer examination suggests that, despite lack of statistical significance, the 50 mg/day dose of vitamin E tended to elevate serum C18:2ω6 and lower serum C20:4ω6. Linoleate in groups 0-T and 50-T was 22.86 $\pm$ 2.61% and 30.96 $\pm$ 4.19%. Arachidonate in groups 1-T and 50-T was 25.02 $\pm$ 1.76% and 18.75 $\pm$ 1.76%, respectively (Table 17).

Expression of the relationships among fatty acids by means of ratios enables one to see more clearly the effects of the α-tocopherol supplementation (Table 19). Essential FA deficiency significantly elevated the C18:1/C18:0 ratio, the C20:4ω6/C18:2ω6 ratio and the

C20:3ω9/C20:4ω6 + C18:2ω6 (T/T + D) ratio. These ratios reflect the Δ9, Δ6 and Δ5 desaturase activities (Rivers and Hassam 1975). Elevation of all three ratios in the EFAD groups suggests enhanced desaturase activities. Removal of the EFAD group from comparison and consideration of 0-T, 1-T, and 50-T suggests that the group of rats receiving the high vitamin E dose had a statistically significant reduction in C20:4ω6/C18:2ω6 ratio, although the other two ratios were unaffected. A reduced C20:4ω6/C18:2ω6 ratio suggests that excessive vitamin E intake altered the balance among these two fatty acids. Calculation of C20:4ω6/C18:2ω6 ratios from serum data of Experiment II yielded values of 0.85, 0.94, 1.02, 0.85, and 0.94 for the 0, 1, 5, 10, and 20 mg/day supplemented groups. Although 20 mg/day vitamin E had no significant effect on serum FA profile, 50 mg/day did alter the relative amounts of C18:2ω6 and C20:4ω6 (Table 19). The mean C20:4ω6/C18:2ω6 ratios in Experiment III were 1.21 for the 1-T group and 0.73 for the 50-T group ($p < .05$). As C20:4ω6 is mostly of endogenous origin, this finding suggests excessive levels of vitamin E may hinder the conversion of C18:2ω6 to C20:4ω6, perhaps through influence on Δ6 desaturase. Another possible explanation is that excess α-tocopheryl acetate protected one fatty acid from oxidative destruction more effectively than the other.

The observation that 20 mg/day vitamin E supplementation did not affect serum fatty acid profiles, while a 50 mg/day dose did produce a change, is consonant with the findings on body weight. A dose of 20 mg/day did not depress growth (Table 9), while 50 mg/day began to depress growth at the end of the experimental period (Figure 8). One concludes that vitamin E intakes up to 40 times the requirement may not be

deleterious to the rat, but intakes at 100 times the requirement may be harmful. This conclusion is based, of course, on only two measures which may be unrelated and may be unimportant to overall health and well-being.

Pulmonary Fatty Acid Pattern

Essential FA deficiency produced a fatty acid pattern in lung similar to, but less pronounced than, the pattern in serum. In Table 24 serum and pulmonary fatty acids in the EFAD group of Experiment II are compared. Lung has a much higher relative amount of C16:0 than serum, which is explicable by the contribution of pulmonary surfactant. Lung has much lower relative concentration of C16:1, C18:0, C18:2ω6, and C20:3ω9 and a slightly lower proportion of C20:4ω6 than serum. Lung has a somewhat higher proportion of C18:1 than serum. The T/T ratio of EFAD lung lipids does not approach the value apparent in serum lipids, suggesting lung tissue is resistant to EFA depletion.

The pulmonary level of C20:4ω6 was similar to the serum level, but C18:2ω6 was nearly fivefold lower in lung than in serum. Clearly, EFA depletion has made inroads, although the simple T/T ratio masks the effect. In Experiment II the serum total lipid T/T ratio was 0.47, while the pulmonary total lipid T/T ratio was 0.14. In Experiment III the pulmonary total lipid T/T ratio was 0.20. Pulmonary C18:2ω6 was again much lower than serum C18:2ω6. In general, replication of pulmonary fatty acid patterns was good between Experiment II and Experiment III EFA depletions.

Table 24. Serum and pulmonary fatty acid profiles after 45 days of EFA depletion. Experiment II

Fatty acid	Serum	Lung
16:0	23.16 ± 1.03[a]	42.90 ± 0.98
16:1	3.38 ± 0.47	1.33 ± 0.55
18:0	22.53 ± 0.97	12.29 ± 0.53
18:1	21.56 ± 1.00	29.17 ± 0.32
18:2ω6	10.46 ± 1.05	2.40 ± 1.18
18:3ω3	Trace	Trace
20:3ω9	6.05 ± 0.66	1.41 ± 0.19
20:4ω6	12.84 ± 1.28	10.48 ± 0.10
T/T	0.47	0.14

[a]Mean ± SEM.

A more complete description of tissue fatty acid patterns than T/T ratio alone gives may be obtained using three additional fatty acid ratios (Table 19). Essential FA deficiency elevated the C18:1/C18:0 ratio compared to essential fatty acid sufficiency. This ratio identifies an enhanced desaturase activity which, in a deficiency of PUFA, reflects an attempt to produce unsaturated fatty acids from the materials at hand. The C20:3ω9/C20:4ω6 + C18:2ω6 (triene/tetraene + diene, T/T + D) ratio is also elevated in lung in EFA deficiency. This ratio provides a more comprehensive statement about EFA status than the T/T ratio for the former also accounts for tissue depletion or retention of C18:2ω6. Serum has a fourfold higher T/T ratio than lung in EFA

deficiency, while lung is more depleted of C18:2ω6 than is serum. Thus, when these two observations are combined in one ratio, the values are 0.40 and 0.17 for serum and lung, respectively. The EFA status of the two tissues is closer than the T/T had indicated (Table 25). Although the lung is still conservative of PUFA in the face of EFA deficiency, the effects of the lack of dietary PUFA are clearly evident.

Table 25. Comparison of indices of EFA deficiency in serum and pulmonary total lipids. Experiment III

	Serum	Lung	Serum/lung
T/T	0.66	0.20	3.3
T/T + D	0.40	0.17	2.4

In Experiment II graded levels of dietary α-tocopheryl acetate had no significant effect on fatty acid pattern in pulmonary total lipid. One has to conclude that dietary α-tocopheryl acetate intakes up to 20 mg/day are of no influence on any of the fatty acids measured. Other investigators have reported similar results (Miller and White 1975, Schoene and Lehmann 1978). Donovan and Menzel (1979) and Donovan et al. (1977) fed α-tocopheryl acetate at 105 mg/kg diet and found no effect on mouse pulmonary fatty acid composition. We provided α-tocopheryl acetate at 1420 mg/kg diet and still found no change in rat pulmonary fatty acid profile due to excess tocopherol. Even when α-tocopheryl acetate supplementation rose to 50 mg/day or 3750 mg/kg diet, no statistically significant differences appeared in C18:2ω6 or C20:4ω6 levels in lung

(Table 18). The C20:4ω6/C18:2ω6 ratio showed no difference in relative amounts of ω6 fatty acids. The animals receiving 50 mg vitamin E daily had relatively no less C20:4ω6 and relatively no more C18:2ω6 in pulmonary total lipids than animals receiving 1 mg vitamin E daily (Table 19). This similarity in relative amounts of C18:2ω6 and C20:4ω6 in lung total lipids despite vitamin E supplement contrasts with the result in serum. While in serum dietary vitamin E seemed to alter the balance of C18:2ω6 and C20:4ω6, the evidence does not support a similar conclusion for lung. Possibly the tissue difference arises from α-tocopherol content of the tissues in question. Although serum α-tocopherol was not measured, there is evidence that serum vitamin E rises in response to diet (Lehmann et al. 1977a, Aftergood and Alfin-Slater 1978). Measures of pulmonary α-tocopherol suggest that lung has only a limited ability to accumulate vitamin E. The lung, therefore, may be shielded from effects of excess vitamin E intake.

The data do not support the conclusion that excess of dietary vitamin E has an influence on pulmonary fatty acid pattern. Many investigators have shown that a deficiency of vitamin E is associated with a decline in C18:2ω6 and a rise in C20:4ω6 (Witting and Horwitt 1967, Lee and Barnes 1969). As yet, an excess of vitamin E has not been associated with any alteration in fatty acid profile. Other investigators have used moderate vitamin E supplementation and for relatively short feeding periods. Donovan and Menzel (1979) fed at most 105 mg dl-α-tocopheryl acetate/kg diet for six weeks, while we fed 3750 mg dl-α-tocopheryl acetate/kg diet for six weeks. A trend to growth depression appeared only in the final two weeks of Experiment

III. Failure of others to observe effects of α-tocopheryl acetate dose may be due to (1) too short an experimental period, (2) too subtle and too moderate a difference in vitamin E treatment, (3) insulation of lung or other tissue from excess vitamin E intakes, or (4) absence of a genuine effect of vitamin E on the variables considered. The results do not provide the final answer but do suggest possibilities that ought to be more fully studied before a firm conclusion can be drawn.

Prostaglandin Synthesis

A reduction in PG synthesis has been observed in EFA deficiency. Our results also suggest that whole blood PG synthetic rate was depressed in EFA deficiency. Comparisons of group EFAD with the other three experimental groups are made with the awareness of an age difference. The EFAD group was six weeks younger than the 0-T, 1-T, and 50-T groups. Glenn et al. (1972) reported that PG synthesis increased with age. Thus, the data may reflect an effect of age rather than diet. Nevertheless, support for reduced PG synthesis in EFA deficiency is found in work by Vincent et al. (1974), Parnham et al. (1979), Weston and Johnston (1978), and others. An apparent reduction in synthesis of PGE_2 and $PGF_{2\alpha}$ in serum was not statistically significant but reduced synthesis of PGE_1 in serum was significant (Table 21). Fine et al. (1980) found that altering dietary P/S ratio from 0.4 to 5.5 had no effect on $PGF_{2\alpha}$ and PGE_2 synthesis in 10-minute incubated whole blood. A general trend of increased PGE_1 synthesis with increasing P/S ratio was seen. Our results support these findings.

The significant reduction in serum PGE_1 and the less pronounced depression of serum PGE_2 and $PGF_{2\alpha}$ by EFA deficiency are a logical consequence of availability of fatty acid precursors. Comparing the EFAD group with the three SO groups reveals C18:2ω6/C20:4ω6 ratios of 0.71 for the former and 1.25 for the latter. What these ratios show is that the amount of C18:2ω6 relative to C20:4ω6 is reduced in serum in EFA deficiency. Although relative concentrations of both C18:2ω6 and C20:4ω6 decline in EFA deficiency, C18:2ω6 drops lower than C20:4ω6. As linoleate is precursor to PGs of the one series and arachidonate is precursor to PGs of the two series, the pattern of PG depression in Table 21 is precisely as expected. Essential FA deficiency depresses synthesis of PGs of the one series more than it depresses synthesis of PGs of the two series.

Although EFA depleted, the animals exhibited a smaller reduction in C18:2ω6 and C20:4ω6 in lung than in serum. Lung was mildly protected from the effects of EFA deficiency, depleting slowly, as evident in both the T/T ratio and the T/T + D ratio. A known inhibitor of PG synthesis, C20:3ω9 was lower in lung than in serum (Tables 17 and 18). Thus, less depression of PG synthesis occurred in lung than in serum. In pulmonary total lipids the level of C20:4ω6 was no lower in EFAD than in the other groups. No reduction of synthesis of PGs of the two series would be expected. While the level of C18:2ω6 was clearly lowered in lung in EFAD, the measured level of PGE_1 was low enough in all groups to obscure any differences. Prostaglandin E_1 may be low in lung due to rapid metabolism.

That pulmonary prostacyclin should be elevated in EFA deficiency

is an unexpected finding. Very few measures of PGI_2 have been reported. Hornstra and Haddeman (1978) reported PGI_2 was decreased in aortic endothelium of EFA deficient animals. Hornstra and Hemker (1979) confirmed the reduction of prostacyclin synthesis in EFA deficiency.

Although the present results appear to contradict these findings, attention to details of the diets used suggests complex factors entering into the comparisons. Hornstra and Haddeman (1978) compared EFA deficiency induced by a fat-free diet with a control diet containing fat. Thus, PGI_2 synthesis may have been reduced by level rather than composition of dietary fat. Hornstra and Hemker (1979) controlled level of fat by comparing rats fed 5% of calories as sunflower seed oil or hydrogenated coconut oil. Although level of fat was consistent, 5% of calories is a low-fat diet. Dupont et al. (1980) showed that the change in rate of PG synthesis in response to change in C18:2ω6 intake was not a simple curve of positive slope. Increasing intake of linoleate from 0 to 2 percent of calories was associated with increased PG synthesis. Increasing linoleate intake from 2 to 7% of calories was associated with a drop in rate of PG synthesis. Increases of linoleate intake over 7% of calories were again associated with increased rate of PG synthesis. Hornstra and Hemker's sunflower seed oil diet was 3% of calories as linoleate. Thus, they were feeding in the area of the PG synthesis curve that Dupont et al. (1980) called "abnormally regulated." Evaluation of the decreased PGI_2 synthesis in EFA deficiency is difficult. It is impossible to conclude from the data presented whether the elevated PGI_2 in our experiment was the result of an artifact, an effect of age or diet, or a combination of

several factors.

Dietary α-tocopheryl acetate was of no effect on PG synthesis in arterial blood or lung homogenate measured after ten minutes of incubation. Lands et al. (1973) reported in vitro inhibition of PG synthesis by α-tocopherol, but Zenzer and Davis (1978) were unable to find inhibition when using concentrations in the incubation medium exceeding those used by Lands et al. Either the inhibition occurred only at low concentration of α-tocopherol or there was no genuine inhibition. In vivo work has not clarified the issue. Likoff et al. (1978) found that PG synthesis in chick spleen was inhibited by feeding a high-vitamin E diet. Our results suggest inhibition of PG synthesis in lung or serum cannot be attributed to excess dietary α-tocopherol intake.

Many effects of excess vitamin E intake have suggested some derangement of PG metabolism – platelet aggregation (Nafstad 1974), altered immune response (Corwin and Shloss 1979), protection from ozone toxicity (Menzel et al. 1978) – but very few measures of PGs in animals or man ingesting excess vitamin E have been reported. More information must be gathered to arrive at a definitive answer. Specific tissues, tissue fractions, species, ages, tocopherol isomers, doses, and individual prostaglandins must be considered.

In theory α-tocopherol could act to inhibit PG synthesis by altering the relative concentrations of fatty acid precursors. Of this we found no evidence in lung. Alpha-tocopherol affected neither fatty acid substrate nor PG product. In serum, while the amount of C20:4ω6 was reduced relative to C18:2ω6 on the 50 mg/day vitamin E supplement, there was no concomitant reduction in PGE_2 and $PGF_{2\alpha}$

relative to PGE_1. As with growth depression, perhaps a longer experimental period would have allowed differences to appear. On the other hand, the experimental period may have been too long already, and any differences were moderated by adaptations over time.

The $PGE_1/PGE_2 + PGF_{2\alpha}$ ratio permits a single, comprehensive comparison of shifting balances among PGs of the one and two series. If α-tocopherol were to alter the balance of C18:2ω6 and C20:4ω6, then one might expect a similar shift in relative amounts of one and two series PGs. Alpha-tocopherol was of no significant effect on PG ratio in lung or serum. Dietary α-tocopherol appears to have no effect on PG synthesis mediated through effect on FA precursors.

Comparison of reported values for PG synthesis is difficult. Method of analysis, diet, tissue, handling, reagent purity all influence results. Samuelsson et al. (1978) discussed the many sources of error in measurement of PGs. Prostaglandins occur in minute quantity such that slight variations in laboratory protocol may have large consequences. Prostaglandins are produced in response to mechanical stimulation. Thus, collection of the sample itself introduces possible error. In our laboratory different batches of antiserum and buffer have been shown to affect the assay results. Incubation of sample allows PG synthesis to proceed, and maximum levels are reached after different periods for different tissues. Incubation permits observation of PG synthetic rate and allows a measure of control over the error introduced in sample collection. Nonincubation of sample permits analysis

of endogenous PG levels. Work is currently underway[1] to examine and compare the levels of PGs measured before and after incubation in a variety of tissues.

A comparison of measured values of PGs using the same RIA procedure appears in Table 26. In arterial serum our PGE_1 values are lower than those of other investigators. This is easily explained by the differences in incubation procedure. Hwang et al. (1975) did not incubate, which should reduce PG values. However, they did not add aspirin to the sample to inhibit further PG synthesis. Prostaglandin levels in samples rise over time in the absence of a nonsteroidal anti-inflammatory drug. Hwang and Kinsella (1978) incubated 60 minutes which permitted more PGE_1 synthesis than our 10-minute incubation. Our serum PGE_2 value is lower than that of Hwang and Kinsella, again due to variation in length of incubation period. Our serum and lung PGE_2 levels are very similar to those of Mathias and Dupont's (1979) which were obtained under identical incubation conditions. Our serum and lung $PGF_{2\alpha}$ values are much higher than any other reported. This is probably an effect of diet. Mathias and Dupont fed only 20% of calories as fat with a P.S ratio of 5.5. We fed 38% of calories as fat with a P.S ratio of 8.6.

Diets high in PUFA have sometimes been shown to have harmful effects. The same diets have been associated with elevated PG synthesis. In addition, vitamin E requirements rise with increasing dietary PUFA, and vitamin E has inhibited PG synthesis in vitro. The hypothesis

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[1] Meydani, personal communication.

Table 26. Comparison of PG levels in rat serum and lung, determined by RIA

	Tissue	Conditions	Diet
Hwang *et al.* 1975	Arterial serum	No incubation No aspirin	20% corn oil
Hwang & Kinsella 1978	Arterial serum	60-min incub.	11% cis 18:2ω6 11% HCO
Mathias & Dupont 1979	Lung	10-min incub.	20% calories as fat P/S 5.5
	Serum	10-min incub.	20% calories as fat P/S 5.5
Work herein	Lung	10-min incub.	20% SO by weight
	Arterial serum	10-min incub.	20% SO by weight

PGE_1	PGE_2	$PGF_{2\alpha}$
1.57 ± 0.23 ng/ml	–	2.77 ± 0.42 ng/ml
5.69 ± 0.59 ng/ml	24.89 ± 4.35 ng/ml	–
1.10 ± 0.24 ng/ml	2.19 ± 0.85 ng/ml	–
–	0.91 ± 0.14 ng/mg	0.50 ± 0.08 ng/mg
–	5.4 ± 1.5 ng/10^9 platelets	3.8 ± 0.6 ng/10^9 platelets
0.08 ± 0.01 ng/mg	0.28 ± 0.02 ng/mg	7.61 ± 0.94 ng/mg
0.69 ± 0.15 ng/ml	5.50 ± 0.99 ng/ml	14.23 ± 2.61 ng/ml

tested by the present investigation was that vitamin E, taken in adequate amounts, could counteract the effects of high PUFA diets by altering PG precursor fatty acids and PG synthesis.

On the basis of the results reported herein, one cannot conclude that increasing intakes of α-tocopherol would ameliorate the deleterious effects due to high PUFA diets through modification of PG synthesis. No benefits from high vitamin E intake and some potential disadvantages were observed. Failure to support the hypothesis does not prove it to be false. The findings may be in error. The variables selected for observation and the methods used may be insensitive. Although the data fail to so indicate, real differences may exist.

SUMMARY

The purpose of this work was to test the interrelationship of dietary vitamin E, dietary linoleic acid, and prostaglandin synthesis. Level of dietary PUFA has been associated with PG synthesis. In EFA deficiency PG synthesis was depressed, and on diets with P/S ratios over 5.5 PG synthesis was elevated. Vitamin E seemed to inhibit PG synthesis in vitro, and vitamin E deficiency in animals was associated with both depressed (Chan et al. 1979) and elevated (Hope et al. 1975) PG synthesis. As the initial step in PG synthesis is formation of a peroxide and as vitamin E is a recognized antioxidant, it has seemed reasonable to expect an influence of tocopherol on PG synthesis. Furthermore, desaturation of linoleate to produce arachidonate (both PG precursors) is an oxidative reaction which may be inhibited by vitamin E. Enhanced platelet aggregation – a function mediated at least in part by prostaglandins – occurs with diets either high in PUFA or deficient in vitamin E. Other effects of high PUFA diets have suggested PG involvement. Requirement for vitamin E is related to intake of PUFA. Thus, the hypothesis for this work was: an increase in dietary α-tocopherol will modify the pulmonary and serum concentrations of precursor fatty acids and will alter PG synthesis. Alpha-tocopherol will prevent conversion of linoleate to arachidonate and thereby alter the balance of PGs of the one and two series.

In order to observe interrelationships of dietary α-tocopherol and dietary linoleic acid, a pre-experimental EFA depletion period was used. In Experiment I male rats were fed a 20% HCO, 26% casein diet in order

to deplete tissue reserves of EFA. Periodic blood samples were taken for determination of T/T ratios, and growth was observed in order to monitor progress of EFA deficiency with time. In Experiments II and III the initial EFA depletion period (45 days) was followed by 45 days of refeeding with 20% safflower oil and graded levels of dl-α-tocopheryl acetate. In Experiment II dl-α-tocopheryl acetate was given in dietary supplements of 0, 1, 5, 10, or 20 mg/day. In Experiment III vitamin E was fed at 0, 1, or 50 mg/day. An EFAD group, killed at the end of the depletion period, was also included in Experiments II and III. Dependent variables observed were weight gain, food intake, hemolysis, pulmonary α-tocopherol, pulmonary and serum fatty acid profiles, pulmonary PGE_1, PGE_2, $PGF_{2\alpha}$, PGI_2, and serum PGE_1, PGE_2, and $PGF_{2\alpha}$. From these measures fatty acid ratios were developed to compare relative amounts of selected fatty acids.

Essential FA depletion had marked effect on fatty acid profiles of serum and lung. Linoleate and arachidonate decreased while eicosatrienoate and oleate rose. The effect was more pronounced in serum than lung. Pulmonary tissue was resistant to, but not untouched by, the deficiency. While T/T ratios of serum were greater than 0.4 within 45 days of beginning EFA depletion, lung T/T ratios were less than 0.20. However, lung showed greater reduction in linoleate than did serum. Thus, a C20:3ω9/C20:4ω6 + C18:2ω6 ratio was proposed that more accurately reflected the depletion of ω6 fatty acids. Use of the T/T + D ratio showed the effects of EFA deficiency on lung and serum to be more similar than the T/T ratio had suggested.

The effect of EFA depletion on PG synthesis was tissue and prostanoid specific, and no generalization was possible. In blood PGE_1

synthesis was depressed. In lung PGI_2 synthesis was elevated. Serum PGE_2, $PGF_{2\alpha}$, pulmonary PGE_1, PGE_2, and $PGF_{2\alpha}$ syntheses were not significantly altered. An explanation for the general absence of depression of PG synthesis is obscure, particularly in view of the clearly reduced levels of PG precursor fatty acids and the elevated level of inhibitory eicosatrienoic acid.

In general it was difficult to see any effect of progressive level of dietary vitamin E on any of the dependent variables. Growth was generally unchanged except for the appearance of a slight growth depression after nearly 45 days of feeding 50 mg dl-α-tocopheryl acetate/day. A very limited accumulation of α-tocopherol in lung was observed. No decrease in percent RBC hemolysis was associated with excessive vitamin E intakes. No alteration in FA profile of serum or lung was obvious with vitamin E intakes up to 20 mg daily. However, in the 50-T group the serum C20:4ω6/C18:2ω6 ratio was significantly lower than in the 1-T group. Such an effect was not observed in lung. This tissue difference was consonant with the accumulation of α-tocopherol in the tissue. Lung had slight deposition of α-tocopherol in response to excessive dietary intakes while serum α-tocopherol is believed to reflect dietary vitamin E.

Increased linoleate relative to arachidonate in serum of the 50-T group supported the hypothesis that vitamin E would retard conversion of linoleate to arachidonate. The effect was not extended to PG synthesis, however. No inhibition of synthesis of PGs of the two series relative to the one series was evident in blood or lung.

In general the data do not support the hypothesis. Alpha-

tocopherol was seen to have little effect on fatty acid pattern of serum and no effect on fatty acid profile of lung. In neither tissue was PG synthesis altered by level of intake of vitamin E. The evidence does not support a beneficial role for vitamin E in high-PUFA diets.

On the other hand, the hypothesis is not proven false. Only selected variables were observed. An effect of dietary α-tocopherol might be evident in other tissues or other functional measures. Moreover, the procedures used in this work may be insensitive to genuine differences. Further efforts on the relationship of vitamin E, fatty acids, and prostaglandins are warranted. Although a zero-supplemented group was included in this research, no evidence of a genuine vitamin E deficiency appeared. In order to determine whether or not vitamin E actually has a part in regulation of fatty acid-prostaglandin metabolism, it is essential to produce a frank vitamin E deficiency and observe the functional consequences. The length of feeding may have masked differences. Thus, future work might feed animals a similar high-PUFA diet and graded levels of vitamin E for three different experimental periods — 21 days, 45 days, and 90 days. The elevated prostacyclin synthesis in EFA deficiency requires confirmation and explanation. The differing responses of prostanoids to the EFA deficient diet suggests a need to avoid generalizations and to observe each prostaglandin, thromboxane, and endoperoxide metabolite for its response to dietary manipulation.

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ACKNOWLEDGMENTS

Heartfelt gratitude is extended to those whose presence has uplifted me and made possible the accomplishment of this goal.

To Dr. Arnrich, who has always treated me with respect and offered judicious guidance.

To Cynthia Shriver, Simin Meydani, Mary Remmes, Jeri Schwabe and others who have rendered invaluable laboratory assistance.

To Cheryl Enger, who aided in statistical analyses.

To Bob, Andrew, Philip, and Nicholas who have loved me unconditionally.

APPENDIX

Table 27. Antisera used for PG-RIA. Experiment III

Antisera	Batch	Date	Working dilution	% binding
Anti-PGE_1	R 1005	10/17/73	1:3000	25%
Anti-$PGF_{2\alpha}$	1002	8/30/73	1:1000	36%
Anti-PGE_2	1010	10/14/73	1:4000	36%
Anti-PGI_2	D-5	10/19/78	1:1000	62%
Normal rabbit serum (NRS)	Pooled 6	10/25/76	1:400	2.6%
Anti-rabbit gamma globulin	Pooled 3	8/11/77	1:20	–

Table 28. Phosphate buffered saline solution (PBS), 0.01M

25 liters pH 7.0

1. Dissolve in approximately 800 ml of distilled water (may require heating):

   a) 204.3 gm NaCl
   b) 9.85 gm $NaH_2PO_4 \cdot H_2O$
   c) 26.7 gm Na_2HPO_4 (anhydrous) or 49.5 gm of 7-hydrate
   d) 2.38 gm Merthiolate (Ethylmercurithiosalicylic Acid Sodium Salt)

2. Dilute to 25 liters in large plastic carboy with distilled water.

3. If pH is not within 6.9-7.1 range, adjust with 20% NaOH or 20% HCl.

4. Store at 4°.

Table 29. Gelatin in phosphate buffer saline solution (PBS-gel), 0.1%

1. Weigh 5 gms of Knox gelatin.
2. Place approximately 600 ml of PBS in a 2-liter beaker. Add the gelatin, stir and heat. When the solution is clear, place in a large plastic container. Dilute to 5 liters with PBS. Mix well. Store at 4°.

Table 30. PBS-EDTA, 0.05M

1. Weigh 18.612 gms disodium EDTA (Ethylenediamine Tetraacetic Acid, FW 372.24, $C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$) in a beaker.
2. Add approximately 800 ml PBS. Warm and stir until dissolved.
3. Bring pH to 7.0 with 5N NaOH.
4. Transfer to 1-liter volumetric flask. When at room temperature, dilute to mark with PBS. Store at 4°.

Table 31. Representative protocol for PG-RIA

Tube No.	µl PBS gel	µl std. or sample	µl antiserum	µl ^{3}H-PG
1	–	–	–	100
2	–	–	–	100
3	–	–	–	100
4	500	–	NRS:ARGG (1:1) 400	100
5	500	–	NRS:ARGG (1:1) 400	100
6	500	–	NRS:ARGG (1:1) 400	100
7	500	–	Antiserum:ARGG (1:1) 400	100
8	500	–	Antiserum:ARGG (1:1) 400	100
9	500	–	Antiserum:ARGG (1:1) 400	100
10	–	500	Antiserum:ARGG (1:1) 400	100
11	250	250	Antiserum:ARGG (1:1) 400	100
12	375	125	Antiserum:ARGG (1:1) 400	100
13	437.5	62.5	Antiserum:ARGG (1:1) 400	100
14	468.8	31.2	Antiserum:ARGG (1:1) 400	100
15	484.4	15.6	Antiserum:ARGG (1:1) 400	100
16	492.2	7.8	Antiserum:ARGG (1:1) 400	100
17	496.1	3.9	Antiserum:ARGG (1:1) 400	100
18	498.0	2.0	Antiserum:ARGG (1:1) 400	100
19	499.0	1.0	Antiserum:ARGG (1:1) 400	100
20	300	200	Antiserum:ARGG (1:1) 400	100
21	300	200	Antiserum:ARGG (1:1) 400	100
22	300	200	Antiserum:ARGG (1:1) 400	100
23	300	200	Antiserum:ARGG (1:1) 400	100
24	100	400	Antiserum:ARGG (1:1) 400	100
25	100	400	Antiserum:ARGG (1:1) 400	100
26	500	–	Antiserum:ARGG (1:1) 400	100
27	500	–	Antiserum:ARGG (1:1) 400	100
28	500	–	Antiserum:ARGG (1:1) 400	100
29	–	500	Antiserum:ARGG (1:1) 400	100
30	250	250	Antiserum:ARGG (1:1) 400	100
31	375	125	Antiserum:ARGG (1:1) 400	100
32	437.5	62.5	Antiserum:ARGG (1:1) 400	100
33	468.8	31.2	Antiserum:ARGG (1:1) 400	100
34	484.4	15.6	Antiserum:ARGG (1:1) 400	100
35	492.2	7.8	Antiserum:ARGG (1:1) 400	100

Table 31. Continued

Tube No.	µl PBS gel	µl std. or sample	µl antiserum	µl ^{3}H-PG
36	496.1	3.9	Antiserum:ARGG (1:1) 400	100
37	498.0	2.0	Antiserum:ARGG (1:1) 400	100
38	499.0	1.0	Antiserum:ARGG (1:1) 400	

Table 32. Balanced incomplete block design. Experiment II

Litter	Treatments assigned			
1	0-T	1-T	5-T	10-T
2	0-T	10-T	20-T	EFAD
3	1-T	5-T	20-T	EFAD
4	0-T	1-T	5-T	20-T
5	0-T	1-T	10-T	EFAD
6	5-T	10-T	20-T	EFAD
7	0-T	1-T	5-T	EFAD
8	0-T	5-T	10-T	20-T
9	1-T	10-T	20-T	EFAD
10	0-T	1-T	10-T	20-T
11	0-T	5-T	20-T	EFAD
12	1-T	5-T	10-T	EFAD
13	0-T	1-T	20-T	EFAD
14	0-T	5-T	10-T	EFAD
15	1-T	5-T	10-T	20-T

Table 33. Influence of genetic variability on dependent variables. Experiment II

Variable[a]	PR > F
Initial weight	.01
Weight gain (days 46-61)	.05
FER (days 1-43)	.01
Serum FA (46th day)	
16:0	.05
16:1	.05
18:0	.05
18:1	.05
18:2ω6	.01
20:3ω9	.01
(91st day)	
16:0	.05
18:0	.01
Lung FA	
16:0	.01
18:1	.01
18:2ω6	.01
20:3ω9	.05

[a]Only those variables exhibiting a statistically significant genetic effect are included.

Table 34. Influence of genetic variability on dependent variables. Experiment III

Variable[a]	PR > F
Initial weight	.01
Weight at end of depletion (45 days)	.01
Weight on 76th day	.01
Weight on 82nd day	.05
Serum FA (91st day)	
C20:3ω9	.05
Lung FA	
C16:0	.05
C18:0	.05
C18:1	.05
C18:2ω6	.05
C20:4ω6	.05
Serum PGs	
PGE_2	.05
Lung PGs	
PGE_2	.05
$PGF_{2\alpha}$	.01
PGI_2	.05

[a]Only those variables exhibiting a statistically significant genetic effect are included.

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